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EDITED BY
SIMON FLEXNER, M.D. PEYTON ROUS, M.D.

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LOCAL SPECIFIC THERAPY OF EXPERIMENTAL PNEUMOCOCCAL MENINGITIS.

III. INCIDENTAL MYELITIS, ABSCESS, AND ORGANIZATION OF EXUDATES.

By FRED W. STEWART, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

PLATES 1 TO 4.

(Received for publication, October 26, 1927.)

During the past year our attention has been directed toward the study of Type I pneumococcal meningitis in the effort to devise some method of therapy which might be hoped to reduce the mortality in this disease. The results of these experiments are described in the first two papers of this series (1, 2). The purpose of the present article is to place on record, mainly photomicrographically, certain features in the pathology of experimental Type I meningitis, occurring late in the course of the disease in dogs, emphasizing thereby some aspects of the experimental infection, which are either rare or else for the most part unnoted in its human prototype.

It early became apparent that a considerable number of the experimental dogs developed central cord lesions. These lesions ranged anywhere from occasional leucocytes in the central canal of the cord, with slight leucocytic invasion of the ependyma and slight perivascular leucocytic infiltration, up through purulent distension of the central canal and adjacent perivascular channels, to, in some instances, widespread destructive myelitis. Similar conditions in human pneumococcal meningitis must be very rare, or else unnoted,—rare probably because the cases are too rapidly fatal to permit their development, or unnoted because cord examinations are infrequent at necropsy unless the clinical data question the possibility of special cord pathology.

Nervertheless, Florand and Nicaud (3) report a terminal pneumococcal myelitis, accompanied by a localized meningitis in a patient with pneumonia and empyema.

It is a noteworthy fact that crisis had occurred with the probable establishment of partial immunity 6 days before the nervous manifestations began, this partial immunity doubtless accounting for the localized character of the meningitis and its duration (25 days). The authors believe the myelitis to have been of vascular origin. Marinesco (4) was able to recover the pneumococcus from a case of myelitis, but we are left in doubt as to whether or not there was a preceding meningitis. Chiari (5) reports a "myelitis suppurativa" complicating bronchiectasis, but the protocol is more suggestive of multiple cord abscesses, possibly embolic in origin; pneumococci were recovered from the lesions.

Despite the apparent rarity of cord involvement, other than meningeal, in human pneumococcal meningitis, it is quite common in dogs. This statement is based upon the gross and microscopic examinations of spinal cords from twenty-six dogs; these animals in some instances were controls, where no treatment was instituted after infection; others died either from accident during treatment, from intercurrent distemper, or of the disease, in spite of treatment. Two were reinfected animals that died after prolonged disease induced by reinfected the meninges some weeks after treatment had resulted in a cure of the primary infection.

The incidence and types of cord involvement in the series of animals examined are shown in Table I. Thus it is seen that seventeen out of twenty-six dogs had purulent material within the central canal of the cord; this consisted sometimes of a few polymorphonuclear and endothelial leucocytes in a mass of precipitated albumin, with occasional strands of fibrin; in other instances the central canal was markedly distended with pus (Fig. 1), so much so that in the fresh gross specimen this pus could be easily expressed by gentle stroking. Paralleling almost, but not quite, the occurrence of pus in the central canal, was a varying degree of leucocytic invasion of the ependyma and of the gray matter immediately surrounding the canal and less often a perivascular infiltration of adjacent vessels; with purulent distension of the canal the perivascular involvement was very marked (Figs. 1 and 3). These cord lesions were found only once in animals dying previous to 42 hours after infection. Severe purulent myelitis with extensive destruction of the spinal cord occurred in but four animals; one of these died at 44 hours; the others succumbed late in the disease. Curiously enough, the severe myelitis in all instances spread into the dorsal half

of the cord, destroying especially the dorsal cornua and only slightly involving the ventral gray matter. Only once did this lesion occur in a treated animal; hence the possibility of its arising from cord trauma is very remote, since no animals were infected subthecally in the cord

TABLE I.

Dog No.	Hrs. after injection	Pus in central canal of cord	Infiltration of region of commissures	Perivascular infiltration	Severe myelitis
1	144	+	+	+	0
2	120	0	+	+	0
3	192	+	+	+	+
4*	114	+	+	+	+
5	90	+	+	0	0
6	90	+	+	0	0
7*	216	+	+	+	+
8	42	+	+	+	0
9	216	+	0	+	0
10	90	+	+	0	0
11	23	0	0	0	0
12	44	Severe myelitis; no trace of central canal			
13	42	+	+	+	0
14	24	0	0	0	0
15	43	Traumatic abscess of cord			
16	114	+	+	+	0
17	90	0	+	0	0
18	16 days	+	0	0	0
19		0	+	+	0
20		+	+	+	0
21†		0	0	0	0
22	42	+	+	+	0
23	114	+	0	0	0
24	42	0	0	0	0
25	66	+	+	+	0
26	18	+	+	+	0

*Reinfected animal.

†Meninges sterile.

region. Conditions are best described by reference to the photomicrographs.

Figs. 1 and 3 are from animals untreated after cisternal infection. The first was a reinfected dog, whose second infection followed 11 weeks after treatment had resulted in the cure of the primary infection.

This reinfected animal survived 8 days, eventually becoming moribund, at which time it was chloroformed. The second dog was given a weak primary infection, remained untreated, and died of the disease on the 8th day. Both cords microscopically show considerable dilatation and are filled with an exudate of polymorphonuclear leucocytes; in one the ependyma is invaded by leucocytes, but is otherwise intact; in the other the ependymal cells are flattened and partially absent and leucocytes are invading the cord parenchyma adjacent to the ependyma. In both cords the blood vessels near the central canal show well marked perivascular accumulations of leucocytes, mainly polymorphonuclears. As invasion of the parenchyma continues, the nerve cells undergo extensive necrosis and are invaded by polymorphonuclear and endothelial leucocytes, the latter becoming large and vacuolated, probably from the ingestion of fatty materials (Fig. 6); the central canal may be completely obliterated by the process in some regions.

Figs. 2, 4, and 5 are from the same cord as Fig. 1; here, however, the process has resulted in what is essentially an abscess burrowing extensively throughout the dorsal gray matter, but only slightly involving the fiber tracts. In certain cords pneumococci have been demonstrated in the sections; indeed, in one instance they were numerous in the gray matter surrounding the central canal and in the canal itself before any significant degree of cellular reaction had manifested itself. Briefly summarized, it may be stated that nearly all dogs suffering from experimental Type I pneumococcal meningitis and surviving beyond the 2nd day of the disease develop some degree of central cord involvement. Such central lesions may progress toward frank suppurative myelitis, and thereby provide foci of disease in no wise amenable to treatment.

Abscesses appear to be uncommon in human pneumococcal meningitis, although cognizance is, of course, taken of the fact that abscesses, for example, of otitic origin, may initiate the process; when we speak of abscesses, we refer to those *following* a primary meningitis. That such may occur, however, is apparent from the reports of Kolmer (6) (cerebellar abscess 4 weeks after meningitis was thought to have been cured) and Wieder (7) (frontal lobe abscess). In the dog abscesses are rare. Fig. 7 shows an abscess of recent origin in the floor of the IVth ventricle; the animal was a reinfected dog and was untreated. Small

cortical abscesses are occasionally seen; they arise either as direct extensions from the meninges *via* the perivascular sheaths, or in infected purpuric hemorrhages; this cortical purpura is a common finding and probably, as a rule, has little significance in abscess formation; for the most part it merely results in collections of endothelial leucocytes filled with blood pigment, the usual reaction to hemorrhage.

It is of interest to inquire into the ultimate fate of the meningeal exudate in animals dying late in the course of the disease, to determine whether or not there is any tendency toward resolution. In all dogs examined, with two exceptions, the process must still be regarded as acute; the two exceptions are animals with fatal reinfections. In both of these animals there is distinct evidence of organization of exudate; this organization is coincident with a change in character of the cellular reaction and the appearance of many lymphocytes, plasma cells, and phagocytic endothelial leucocytes. The fibrin network thickens and shrinks and is invaded by endothelium and fibroblasts (Fig. 9); this organization by endothelium and fibroblasts parallels in intensity the distribution of fibrin. The appearance of many chronic inflammatory cells in the exudate produces a very peculiar alteration in sections, particularly in the regions of the choroid plexuses, superficial penetrating vessels, and occasionally in the central cord lesions; there is little or no increase in the actual number of vessels, but a tremendous perivascular thickening well shown in Fig. 8, where every vessel in the choroid plexus is surrounded by a darkly staining zone of chronic inflammatory cells, outside of which the acute character of the exudate is still apparent.

In these chronic, perivascular, inflammatory zones may be observed a decided increase in what Kubie (8) describes as the clasmatocytes of the adventitial sheath, which he has found to multiply under conditions of stimulation, such as, for example, herpetic encephalitis. This clasmatocytic stimulation produces marked perivascular thickening; in certain vessels the clasmatocyte increase appears to be independent of the vascular endothelium, the latter being traceable as a thin, endothelial lining beneath thickened layers of sheath clasmatocytes; in other instances the endothelium seems to take part in the process and pictures such as that shown in Fig. 10 result, where no distinction appears between the endothelium and perivascular cells, suggesting,

therefore, that the swollen endothelial cells may be contributing to those of the adventitial sheath. Such supposition gains confirmation from numerous examples of processes, such as those apparent in Figs. 13 and 14, the former showing a diaster in the thickened endothelial cell, with surrounding perivascular clasmatocytic increase, the latter displaying two mitoses, one outside of the endothelium, the other apparently splitting off toward the vascular channel. Figs. 11 and 12 both demonstrate mitoses in perivascular spindle-shaped cells, closely applied to the endothelium, but nevertheless distinctly separate. Kubie was unable to detect the participation of endothelium in the increase of adventitial clasmatocytes, but the stimulation was doubtless less severe and the process less active in tissues studied by him.

SUMMARY AND CONCLUSIONS.

1. In experimental Type I pneumococcal meningitis of dogs central cord involvement is common after the 2nd day of the disease.

2. These central cord lesions may progress toward extensive suppurative myelitis.

3. Abscesses are infrequent; they arise either as direct perivascular extensions of the meningeal disease or at the site of infected purpuric lesions.

4. In prolonged untreated meningitis of reinfected animals, nothing resembling the disappearance of exudate following crisis in pneumonia occurs. The tendency is toward organization.

5. Late lesions are characterized by the marked development of a perivascular clasmatocytic apparatus, similar to, but more extensive than that hitherto reported in experimental herpetic encephalitis.

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EXPLANATION OF PLATES.

PLATE 1.

FIG. 1. Spinal cord. Distended central canal, destruction of ependyma, leucocytic invasion of gray matter in region of commissures. $\times 110$.

FIG. 2. Acute suppurative myelitis. $\times 18$.

FIG. 3. Spinal cord. Pus in central canal, invasion of ependyma, marked perivascular infiltration. $\times 130$.

PLATE 2.

FIG. 4. Acute suppurative myelitis. $\times 18$.

FIG. 5. A higher power view of the same process. $\times 160$.

FIG. 6. Necrotic anterior horn cells, inflammatory cells, many fatty endothelial leucocytes. $\times 320$.

PLATE 3.

FIG. 7. Abscess of medullary floor. $\times 18$.

FIG. 8. Choroid plexus, showing marked perivascular thickening. $\times 18$.

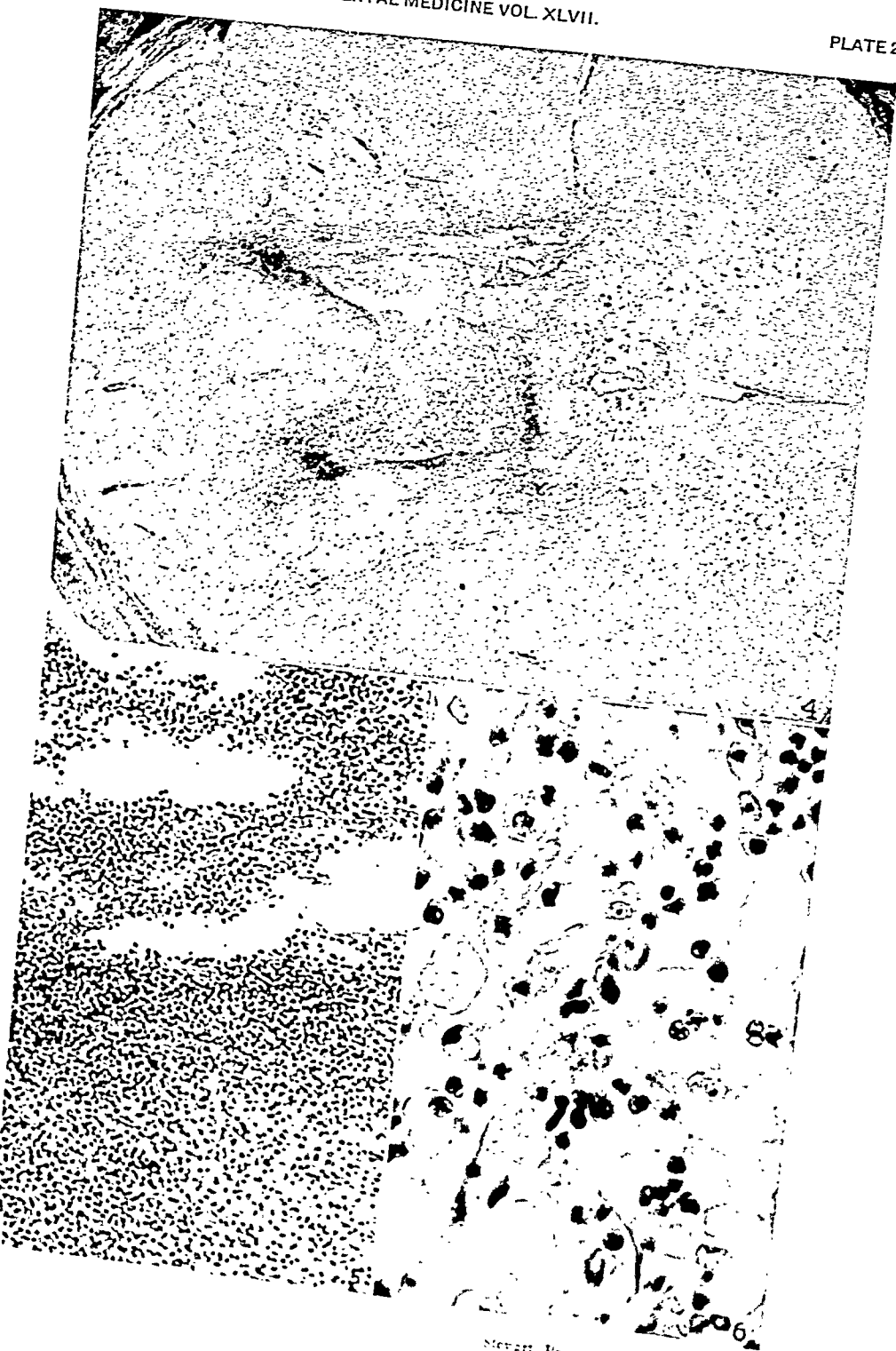
FIG. 9. Organization of fibrin in choroid plexus late in the course of a reinfection meningitis. $\times 570$.

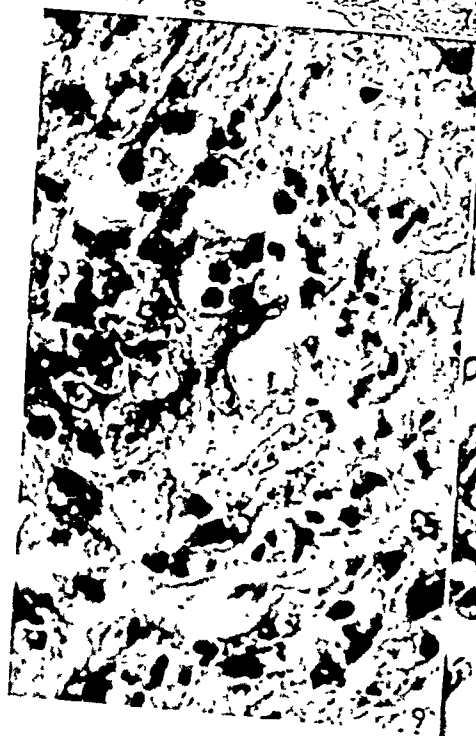
FIG. 10. Increase of adventitial clasmatocytes, suggesting a reduplication of the endothelium. $\times 1000$.

PLATE 4.

FIGS. 11 to 14. Endothelial and perivascular clasmatocyte proliferation, giving rise to the thickened perivascular phagocytic apparatus. $\times 1000$. (*a* = mitotic figures.)









CONTRIBUTIONS TO THE PATHOLOGY OF EXPERIMENTAL VIRUS ENCEPHALITIS.

IV. RECURRING STRAINS OF HERPES VIRUS.

By SIMON FLEXNER, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, September 28, 1927.)

Anyone wishing to inform himself of the present state of our knowledge of the subject of virus encephalitis¹ would do well to read the two comprehensive reviews of Doerr.² While these reviews deal chiefly with herpes virus, they touch on the related subject of so called encephalitis virus as well. In this way, the etiology of epidemic encephalitis is also brought under consideration. While Doerr inclines to the view that epidemic encephalitis in man is a form of herpes virus infection, yet he presents an impartial because quite complete discussion of the many sides of this important topic of controversy.

Our studies, to which those to be recorded in this paper relate, have dealt with the encephalogenic and other properties of the herpes virus, because Flexner and Amoss³ were led to an investigation of this virus through experiments regarding the etiology of epidemic encephalitis. The conclusions reached were in conflict with those previously announced by Doerr² and by Levaditi.⁴ The studies on the etiology of encephalitis have been continued and the present papers are intended to record the results of the more recent experiments. These later studies support the view previously put forward, namely that herpes virus encephalitis and epidemic encephalitis are definite pathological affections, etiologically distinct.

¹ Flexner, S., and Amoss, H. L., *J. Exp. Med.*, 1925, xli, 231.

² Doerr, R., *Zentr. Haut- u. Geschlechtskrankh.*, 1924, xiii, 417; 1924-25, xv, 1, 289; 1925, xvi, 481; *Centr. Bakt., I. Abt., Orig.*, 1925-26, xcvi, suppl., 76.

³ Flexner, S., and Amoss, H. L., *J. Exp. Med.*, 1925, xli, 215, 233, 357. Flexner, S., *J. Am. Med. Assn.*, 1923, lxxxi, 1688, 1785.

⁴ Levaditi, C., *L'herpès et le zona*, Paris, 1926.

severe secondary herpetic cerebral inflammation, and the successive inoculations in series indicate that cornea to cornea passages of the H.F. II virus lead to moderation, not to intensification, of its pathogenic action.

Brain and Cord Passages.

In order to compare the mode of action, or virulence, of one strain of herpes virus with another, it is desirable to submit the strains tested to essentially the same treatment. We have observed that a strong, but not a weak, strain of herpes virus when obtained from the brain of a succumbing rabbit is more likely to produce fatal encephalitis on corneal inoculation than the same virus derived from mere corneal passages. Why this difference should arise, is not known; and whether it is merely a quantitative effect, or has to do with a qualitative change in the strain, has not been determined. It will be recalled that the original H.F. I strain of virus, obtained by subdural inoculations of the rabbit, when subsequently instilled into the eyes produced keratoconjunctivitis followed by fatal virus encephalitis in all of the fifteen rabbits inoculated.

The H.F. II virus injected subdurally in rabbits regularly induces stormy symptoms of virus encephalitis and brings about death in from 4 to 6 days. Characteristic histological lesions and inclusion bodies are found on microscopic examination of the brain tissue. But when the infected brain is used for corneal inoculation, a difference appears in that, contrary to the fatal effects of H.F. I virus under these circumstances, recovery ensues from the inflammation induced by H.F. II virus.

Brain to Cornea Passage.—Three protocols are given, in order to show that while the injection of H.F. II vesicular fluid directly into the brain induces fatal virus encephalitis, the brain to cornea passage of the virus which induces severe keratoconjunctivitis sets up only transient brain symptoms.

Rabbit I.—Mar. 30, 1926. 0.1 cc. of salt solution suspension of herpes vesicular fluid injected intracerebrally.^a Mar. 31. Temperature 41.8°C.; urine reten-

^aAll operations were performed under ether anesthesia.

tion. Apr. 1. Temperature 41.4°C .; tremor. Apr. 3. Tremor increased. Apr. 4. Salivation; gnashing; convulsions; prostration; death.

Rabbit II.—Apr. 6, 1926. Cocainized right eye scarified with cataract knife dipped in salt solution suspension of brain of Rabbit I. Apr. 8. Eye closed, purulent, and vesicles along incisions. Apr. 9. Inflammation severe. Apr. 12. Inflammation subsiding; cornea opaque; circling to right. Apr. 14. Eye clearing; no circling. Recovery was finally complete.

Rabbit III.—Apr. 6, 1926. Right eye treated as with Rabbit II. Apr. 8. Eye closed; severe keratoconjunctivitis; vesicles along incisions. Apr. 8-14. Gradual abatement of inflammation and clearing of the eye. No circling movements seen. Recovery took place.

This experiment may be taken as indicating a difference in quality in two herpes virus strains derived from a supposed virus carrier, that is, a subject of recurrent labial herpes, taken at an interval of 4 years. The next experiment, in which indirect brain, spinal cord, and corneal passages were made, brings out the same general fact, although it shows that the H.F. II virus is capable at times, in passing from the cornea to the brain in sufficient concentration, to set up fatal virus encephalitis.

Brain to Brain to Cornea Passages.—Although the direct passage of the H.F. II virus from brain to cornea failed to produce keratoconjunctivitis followed by encephalitis and death, yet if the virus is passed successively through the brain, it is capable of setting up fatal encephalitis upon corneal inoculation. For example, after the fourth cerebral passage, in which death occurred respectively on the 4th, 6th, 4th, and 4th days, the symptoms having in all instances been characteristic of virus encephalitis, a rabbit was inoculated into the cocaineized right eye, the cornea of which had been scarified. The protocol of the test follows.

Rabbit.—May 5, 1926. Eye inoculation with brain virus H.F. II, 4th brain passage, carried on cataract knife used for scarification of cornea. May 7. Eye closed; suppuration; vesicles along incisions. May 9. Circling to right side. May 12. Temperature 41.9°C .; circling; hypersensitive; retention of urine. May 13. Circling; falls to right side; gnashing; salivation. May 14. Convulsions; death.

Indirect Brain, Cord, and Corneal Passage.—The protocols which follow relate to skin to spinal cord, cord and brain to cornea, and cornea to cornea passages.

Rabbit I.—Apr. 13, 1926. Right side of animal shaved and three scratches penetrating into cutis made; a 30 per cent suspension of fresh brain of rabbit of 2nd brain passage rubbed into scratches. Apr. 16. Local inflammation and vesicle formation. Apr. 19. Temperature 40.6°C.; animal moves about slowly. Apr. 21. Paralysis of hind extremities and back; retention of urine. Apr. 22. Fore legs paretic; other symptoms persist. The moribund animal etherized and the pons and lumbar spinal cord employed separately for corneal inoculations.

Rabbit II.—Apr. 22, 1926. Usual corneal inoculation with suspension of pons of preceding animal. Apr. 24. Slight inflammation; no suppuration. Apr. 26. Vesicles along incisions; slight suppuration. Scrapings taken for corneal inoculation of Rabbit IV. Apr. 27. Eye closed; purulent secretion; more pronounced vesiculation. May 1. Tremor. May 2. Temperature 41.2°C.; tremor; convulsions; urine retention. Death occurred on this date.

Rabbit III.—Apr. 22, 1926. Usual corneal inoculation of right eye with suspension of lumbar spinal cord of Rabbit I. Apr. 25. Eye closed; suppuration; vesicle formation. Apr. 27. Circles to right. May 1. Tremor; retention of urine. May 2. Temperature 40.8°C.; convulsions followed by prostration. Death on this date.

Rabbit IV.—Apr. 26, 1926. Right corneal inoculation with scrapings taken from cornea of Rabbit II. Apr. 28. Eye closed; suppuration; vesicle formation. May 1. Circles to right. May 3. Temperature 41.2°C.; tremor; retention of urine. May 5. Convulsions; prostration; death.

With the results of the foregoing experiments before us, we are prepared to place the H.F. II virus among the strains of herpes virus which are dermatotropic, rather than neurotropic in property. This strain of virus is of medium pathogenicity. It tends to produce local inflammation of the cornea and conjunctivæ, and to invade the brain to a slight degree only; and yet, when implanted on the scarified skin and especially when passed successively through the brain, it acquires neurotropic properties of such invasive power as to suffice to induce fatal encephalitis on corneal implantation. Skin inoculation of the virus gives rise to myelitis, with diffusion of the virus throughout the central nervous organs. Although the test is not conclusive on the point, yet the concentration of the virus in the lumbar cord appeared to be greater than in the pons under these circumstances.

A comparison of the activities of H.F. I virus obtained in 1922 with H.F. II virus secured in 1926 from a subject of recurrent labial herpes shows quite conclusively that the pathogenic activity as measured in the rabbit differs. H.F. I virus is predominantly neurotropic, while H.F. II virus is predominantly dermatotropic. Perhaps the isola-

tion and study of a third strain of the virus may throw light on the nature of the variation.

II.

In addition to the tests carried out with the H.F. II strain of herpes virus, two other specimens of virus from subjects of recurrent herpes were studied. One, called T. strain, was derived from a labial vesicle of a female adult suffering from common cold, and the other, called F. strain, from a child of 4 years who passed at intervals of a few months through attacks of slight fever attended by obscure, nervous disturbances slight in degree, and labial herpes. As will appear, the T. and F. viruses stand at extreme scales of pathogenic action, the former being highly and the latter hardly at all neurotropic for the rabbit.

Corneal Passages, T. Strain.

Three passages from cornea to cornea were carried out with the T. strain, as shown in the following protocols. The course of the pathological process was essentially the same in all, consisting of severe keratoconjunctivitis, encephalitis, and death.

Rabbit I.—Sept. 30, 1926. Right eye cocaineized and cornea scarified with cataract knife dipped in salt solution containing small quantity of clear exudate of lip herpes vesicle, about 12 hours old. Oct. 2. Inflammation; watery exudate; vesicles along incisions. Oct. 4. Eye closed; purulent exudate. Material taken for inoculation into Rabbit II. Oct. 5-7. Inflammation of eye increased; animal refuses food. Death.

Rabbit II.—Oct. 4, 1926. Usual method of inoculation of right eye after scarification of cocaineized cornea with exudate taken on 4th day from Rabbit I. Oct. 6. Eye closed; purulent exudate; vesicle formation. Oct. 8. Temperature 40.6° C.; circles to right. Oct. 9. Circling increased; twists body and falls to right side; gnashing. Oct. 10. Temperature 41.3° C.; convulsions; falls. Oct. 11. Prostration and death.

Rabbit III.—Oct. 11, 1926. Usual method of inoculation of right eye after scarification of cocaineized cornea with exudate from eye of Rabbit II. Oct. 15. Temperature 40° C.; eye closed; purulent exudate. Oct. 17. Temperature 41.2° C.; tremor and rapid circling to right side. Oct. 19. Temperature 41.4° C.; hypersensitive, rapid respiration; retention of urine. Oct. 20. Symptoms continue; falls to right and recovers feet with difficulty. Oct. 21. Profuse salivation; convulsions; death.

Brain to Cornea Passages, T. Strain.

A parallel series of inoculations to the former was carried out with the T. virus, in which the original, diluted vesicular contents were inoculated intracerebrally into Rabbit I, and the brain virus thus secured was used to inoculate the cornea of Rabbit II. Rabbit I developed virus encephalitis and succumbed on the 9th day; Rabbit II first developed keratoconjunctivitis, then showed signs of encephalitis on the 6th day, and succumbed to the latter on the 9th day. A third rabbit was inoculated in the cocaineized, scarified eye from the brain virus of Rabbit II of the cornea to cornea series. The succession of events was typical: gradual development of keratoconjunctivitis, involvement of the brain, with symptoms of tremor, circling, falling, salivation, and death on the 12th day.

The strain of F. virus was studied in detail, as will appear from the summary of experiments to follow, in the course of which a certain number of disputed points were dealt with and perhaps elucidated.

Cornea to Cornea Passages, F. Strain.

A salt solution suspension of the contents of the labial herpetic vesicle was inoculated by the usual method, that is, by dipping the cataract knife into the suspension and then scarifying the cocaineized right cornea of two rabbits with it. Keratoconjunctivitis developed promptly in both animals, and ran the usual course attended by recovery. No symptoms of brain involvement appeared; the highest temperature recorded was 40.7°C., which was reached in both animals.

At the height of the local inflammation (3rd day), exudate was used to inoculate the next two rabbits, constituting the second passage of the series. The events resembled those of the first passage, except that in both animals circling to the right appeared on the 3rd and 4th days respectively. About the 9th day after inoculation the circling abated or ceased, after which recovery was uninterrupted.

In all, ten cornea to cornea passages were made. In no instance was a fatal virus encephalitis induced, and in no case did other signs of brain involvement than that of circling appear. When both eyes were inoculated no circling took place, although the inflammation

produced was typical and severe. Temperatures as high as 41.1°C . arose independently of cerebral symptoms. In the later passages, seventh to tenth, the local reaction was less severe, the temperature lower, and recovery more rapid. Although one eye only was inoculated, no circling occurred.

The conclusions we have drawn from this series of inoculations are:

1. The typical herpes virus keratoconjunctivitis in the rabbit may be attended by fever.

2. Circling to the inoculated side may appear without any other signs of cerebral invasion of the virus.

3. Circling does not appear when the two eyes are inoculated.

4. The virus diminishes in activity in successive corneal passages, as indicated by feebler inflammatory reaction, shorter duration of the inflammation, and absence of circling movements.

Brain and Spinal Cord to Cornea.

The F. virus, as stated, is a weak strain. Tests were carried out in order to determine whether intracerebral injections induced virus encephalitis. It was found as a rule that fatal encephalitis ensued when eye exudate or brain virus was thus injected, but that recovery might occur after intracerebral inoculation.

Rabbit.—Mar. 15, 1926. Animal received intracerebral inoculation of fresh rabbit brain virus. Mar. 19. Temperature 41.1°C .; tremor; retention of urine. These symptoms persisted for 3 days, then abated, and recovery followed.

Experiments were also made in order to determine whether the F. brain virus introduced into the cornea induced both keratoconjunctivitis and frank encephalitis. As a matter of fact, the inflammation of the eye induced is severe, while the effect on the brain is mild only, and if permitted, recedes leaving the rabbit to all intents and purposes normal. And yet if the affected rabbit is killed at the right moment, the virus is detectable by inoculation in the brain tissue.

Rabbits.—Two rabbits, A and B, were given eye inoculations with the fresh F. rabbit brain virus on Sept. 8, 1927. In both, severe keratoconjunctivitis followed. Rabbit A showed fever (40.6°C .) and the head turned to the inoculated (right) side on Sept. 13. On Sept. 15, circling was noted. Killed on this date.

and two rabbits, C and D, were given intracerebral injections of suspension made from the pons.

Rabbit B developed similar symptoms to Rabbit A and was permitted to recover.

Rabbits C and D were given cerebral inoculations with the pons of Rabbit A on Sept. 16. Typical virus encephalitis with characteristic symptoms arose on the 5th and 6th days, and death resulted in both instances on the 9th day.

Since the F. virus is a weak strain, the preceding experiment does not always succeed. In one example, two rabbits were given eye inoculations with material taken from an eye on the 4th day of the keratoconjunctivitis. Severe inflammation attended with circling developed. On the 6th day of the circling, one of the rabbits was killed and the brain used to make a corneal inoculation in one animal, and cerebral inoculation in two animals. No effects followed. At this later period in the encephalitic process, the virus was no longer demonstrable by the inoculation test.

The conclusion to be drawn from these tests is that the F. virus is only weakly neurotropic, and while capable of penetrating to the brain of rabbits, tends to be suppressed there. In this respect, the rabbit treats a weak virus in a manner resembling the way in which the guinea pig also deals with a weak virus.⁹ When the quantity of a weak strain reaching the brain of the rabbit is not excessive, it can be destroyed; and yet, this same weak strain when injected in quantity, is capable of producing fatal encephalitis. As is to be expected, a stronger virus, such as the H.F. II strain, is even more readily detected in the pons after eye infection.

Skin to Spinal Cord.

Although the F. virus does not pass from the cornea to the brain in sufficient concentration to produce fatal virus encephalitis, yet it is capable of passing from the skin to the spinal cord in a way to induce paralysis and probably death.

Rabbit.—Mar. 9, 1926. Right side of body shaved, scratched, and covered with 30 per cent suspension of rabbit brain virus taken 5th day after inoculation. Mar. 12. Mild dermatitis. Mar. 15. Dermatitis and formation of vesicles. Temperature 40.9°C. Mar. 17. Curvature of spine; hind legs paralyzed; reten-

⁹ Rose, G., and Walthard, B., *Z. Hyg. u. Infektionskrankh.*, 1925-26, cv, 645.

tion of urine. Mar. 18. Temperature 40.8°C.; moves fore legs; almost prostrate; etherized.

Rabbit.—Mar. 10, 1926. Right side of body shaved, scratched, and covered with rabbit brain virus of 6th day. Mar. 13. Dermatitis and vesiculation. Mar. 17–18. Dermatitis subsiding; temperature 40.8°C.; spinal curvature; hind legs weak. Mar. 19–20. Paralysis of back and hind legs complete; tremor; etherized.

Rabbit.—Apr. 8, 1926. Right side of body shaved, scarified, and covered with rabbit brain virus of 7th day. Apr. 11. Dermatitis and vesiculation. Apr. 13. Inflammation subsiding. Apr. 14–15. Temperature 41.3°C.; back muscles and hind legs weak. Apr. 16–21. Complete paralysis of posterior half of body; tremor; etherized.

Immunity.

The experiments with H.F. II virus yielded a number of rabbits in which recovery took place from the corneal inflammation. The animals were subjected to immunity tests in a twofold manner, namely by way of corneal and of intracerebral inoculation. It developed that while the previously uninoculated cornea proved to be but partially protected from infection and inflammation, the brain test showed complete immunity of that more sensitive organ, a result in conformity with observations made by Rose and Walthard.⁹ The protocols will be given in pairs, in order to bring out this interesting fact. The brief interval elapsing between the two protection tests is, according to usual measures, insufficient to produce general immunity. As the reinoculations in each set were made on the same day, single control rabbits sufficed for each. Thus it can be stated in advance that the control for the corneal tests developed severe keratoconjunctivitis followed by symptoms of severe virus encephalitis, while the control for the intracerebral tests succumbed to typical virus encephalitis on the 6th day.

Test I. Rabbit.—The corneal test was made 3 months after the first corneal inoculation, and the brain test 12 days after the corneal test. July 19, 1926. Left cocaineized, scarified cornea inoculated with 5 per cent suspension of fresh rabbit brain virus. July 22. Eye closed; purulent exudate; small vesicles. July 26. The inflammation gradually subsided. No symptoms of brain involvement. July 29. Eye clear. July 31. Injected intracerebrally 0.1 cc. 5 per cent suspension of fresh rabbit brain virus. No symptoms of any kind followed.

Test II. Rabbit.—In this instance the interval between the two corneal inoculations was 2½ months. July 19, 1926. Left, cocaineized, scarified cornea inocu-

lated. July 22. Purulent inflammation and vesicle formation. July 23-27. Eye closed; purulent exudate; then gradual clearing. July 31. Eye clear. Injected intracerebrally with 0.1 cc. of 5 per cent suspension of fresh brain virus. No symptoms of any kind followed.

Test III. Rabbit.—The interval between the first and second corneal inoculations was about 10 weeks. July 19, 1926. Inoculated left, cocainized, scarified cornea. July 22. Eye closed; purulent exudate; vesicles formed. July 28. Eye open and clear. July 31. Intracerebral inoculation of 0.1 cc. 5 per cent suspension of fresh brain virus. No symptoms.

In order to complete the account, it remains to mention the simultaneous tests by intracerebral inoculation of two rabbits which had received corneal inoculations $3\frac{1}{2}$ to $4\frac{1}{2}$ months earlier respectively. The $3\frac{1}{2}$ month animal succumbed to virus encephalitis; the other remained free of symptoms.

Besides the H.F. II animals tested for immunity, five rabbits which had been given corneal inoculations of the F. virus 5 or 6 months before were, along with a control, reinoculated in the left (unused) eye. The point of interest is that while all six rabbits developed keratoconjunctivitis from which they recovered, only the control animal showed the circling symptom.

SUMMARY.

In this paper, three strains of the herpes virus have been dealt with. The H.F. II strain was obtained from the subject H.F. 4 years after the H.F. I strain was secured. H.F. is a victim of recurrent herpes. If the subject is also a chronic carrier of the herpes virus, then it is not one, but two or more strains which are persistently carried. The H.F. II strain is of mitigated pathogenic action for the rabbit, as compared with the H.F. I strain; it is to be classed as dermatotropic rather than neurotropic. And yet, in the subject there was no indication that the attack of herpes provoked was different from the other attacks associated with the H.F. I virus.

The other two herpes strains derive their interest from the fact that they came also from persons who suffer from repeated attacks of labial herpes. One strain proved highly neurotropic, resembling in this respect the H.F. I strain; the other was hardly neurotropic at all, but was none the less definitely dermatotropic. It may be possible

at a later date to secure other samples of virus from these individuals for comparison. The dermatotropic F. strain penetrates to the central nervous system far more readily and certainly from the skin than from corneal surfaces.

The recovered inoculated rabbits showed only relative protection to reinoculation of the herpes virus. A notable difference appeared in the degree of protection acquired, on the one hand by the cornea and on the other by the brain. While the one was partial, the other was complete. The complete resistance of the brain was shown (a) by the complete failure of the intracerebral inoculation, and (b) by the absence of circling movements following corneal inoculation.

CONCLUSIONS.

Subjects of recurrent labial herpes may yield more than one strain of the herpes virus.

While the H.F. I strain is notably neurotropic, the H.F. II strain, obtained 4 years later, is slightly neurotropic and strongly dermatotropic for rabbits.

The neurotropic property of the H.F. II virus is somewhat increased by brain passage.

Dermal inoculation of the H.F. II strain leads to myelitis, with extension of the virus to the brain. The concentration of the virus in the lumbar cord seems greater than in the pons.

The T. specimen of the herpes virus is apparently of maximal neurotropic potency for rabbits.

The F. specimen of the herpes virus is of low neurotropic and moderate dermatotropic activity. Passage from eye to eye tends to diminish the effect of the virus. When the F. strain is inoculated into one eye, circling occurs; when into both eyes, circling does not occur. None of the corneally inoculated F. rabbits succumbed to virus encephalitis. And yet, the F. virus exists in the brain of the corneally inoculated rabbits and can be detected there, by cerebral inoculation, on the 1st or 2nd day, but not on the 6th day of the circling. When the F. virus does not reach the brain in excessive amounts, it is suppressed there; when injected in large quantity, it induces fatal encephalitis.

The rabbit brain possesses the power of destroying weak strains of the herpes virus in a manner not dissimilar to that possessed by the guinea pig brain.

Immunity tests showed that in rabbits previously inoculated into the cornea, the opposite cornea is only partially, while the brain is wholly, protected against reinoculation effects. The partially protected rabbits developed on corneal reinoculation local lesions, but unlike the control animal, did not show circling movements.

CONTRIBUTIONS TO THE PATHOLOGY OF EXPERIMENTAL VIRUS ENCEPHALITIS.

V. HERPES VIRUS ENCEPHALITIS IN THE GUINEA PIG.

BY SIMON FLEXNER, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

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The attempt has been made a number of times to ally epidemic encephalitis in man and herpes virus encephalitis in the rabbit. Ever since Doerr and Vöchting's¹ important observation that rabbits inoculated intraocularly sometimes develop fatal herpes virus encephalitis, this point of view has been in the background. Within a year or two, the view has come more into the foreground of the investigative studies. In two sets of investigations especially, namely those of Levaditi and his coworkers² and Doerr and his associates,³ it has been sought to uphold this view-point. Flexner and Amoss have dealt with Levaditi's contention in another place.⁴ It is proposed in the present paper to deal with Doerr's recently published views based on the tests with guinea pigs carried out and described by Rose and Walthard.⁵

As is well known, nothing is easier than to implant the virus contained in herpes vesicles on the rabbit, and to carry the virus in indefinite passages from rabbit to rabbit.

On the other hand, it has proven extremely difficult to implant such a virus on rabbits with material taken from cases of epidemic encephalitis in man. The several hundred or more transfers of these

¹ Doerr, R., and Vöchting, K., *Rev. gén. ophth.*, 1920, xxxiv, 409.

² Levaditi, C., and Nicolau, S., *Compt. rend. Soc. biol.*, 1924, xc, 1372. Levaditi, C., Nicolau, S., and Poincloux, P., *Compt. rend. Soc. biol.*, 1924, xc, 1376. Doerr, R., *Centr. Bakt., I. Abt., Orig.*, 1925-26, xcvi, suppl., 76.

³ Doerr, R., *Centr. Bakt., I. Abt., Orig.*, 1925-26, xcvi, suppl., 76.

⁴ Flexner, S., and Amoss, H. L., *J. Exp. Med.*, 1925, xli, 215.

⁵ Rose, G., and Walthard, B., *Z. Hyg. u. Infektionskrankh.*, 1925-26, cv, 645.

materials from man to rabbit have yielded, as Flexner⁶ has pointed out and Doerr³ concedes, six successful inoculations at most. The percentage of successes is almost minimal. The matter at issue is the explanation of the disparity, the burden of proof being of course placed upon those investigators who would identify the herpes virus with the supposedly microbic incitant of epidemic encephalitis.

An explanation is called for all the more emphatically because herpes eruptions carrying virus are so common and ubiquitous an affection of man. The eruptions occur under the most varied circumstances—as attendants of severe disease and as accompaniments of trivial ailments. To ascribe, therefore, to this almost uniformly and universally present—nearly innocent—pathological material the sinister rôle of the inciting microbic agent of epidemic encephalitis is a position not to be lightly assumed or accepted. It does seem rather beside the point to cite, as Doerr³ has done, as accounting for the irregularity of the infection of rabbits with inocula from cases of epidemic encephalitis, the instance of typhus fever, in which only one-half of the inoculations are successful; or, as has even more recently been urged, the fact that diphtheria bacilli may be carried by persons who have not had and do not acquire diphtheria.³ The evidences for the existence of a virus of typhus fever are not of this hypothetical order, and those for the specific pathogenic effects of the diphtheria bacillus rest upon a pyramid of knowledge with which the fragile structure of the herpes virus etiology of epidemic encephalitis cannot properly be compared.

The foregoing statement is important only because the undertaking to support the thesis of the herpes virus origin of epidemic encephalitis has recently taken a new turn. It has long been known that while rabbits are highly subject to herpes virus infection, other rodents are far less susceptible to its inoculation. And yet rats, mice, and guinea pigs can be infected, although they often survive even subdural injection of the virus. Recently the guinea pig has been submitted to renewed study under circumstances which have yielded results of a kind to inspire the investigator with new ardor in pursuing the hypothetical relationship between the supposed virus of epidemic encephalitis and the virus of febrile herpes.

⁶ Flexner, S., *J. Am. Med. Assn.*, 1923, lxxxii, 1688, 1785. Flexner, S., and Amoss, H. L., *J. Exp. Med.*, 1925, xli, 215.

As so often happens, two papers dealing with the subject appeared so near together that they may be regarded as having had an almost simultaneous origin. The first was published by Dmitrieff,⁷ who states that he chanced on the observation that a strain of herpes virus when introduced into the brain of the guinea pig is quickly reduced in virulence. A study of his protocols shows that even in the first guinea pig transfer this reduction, amounting virtually to destruction, took place. The source of the virus was a brain taken from a rabbit which succumbed to intracerebral inoculation in 3 to 6 days. The rabbit brain material produced encephalitis in young guinea pigs, ending fatally on the 10th or 11th day. When the brains from these guinea pigs were reinoculated into rabbits or other guinea pigs, no obvious effect was produced. When the original rabbit brain virus was injected subdurally into older guinea pigs, either mild symptoms followed by recovery ensued, or no symptoms whatever arose. In one recorded instance the rabbit virus induced keratoconjunctivitis in a guinea pig, and material from the inflamed eye produced, on intracerebral injection, fatal encephalitis in the rabbit.

Although the experiments were few in number, the deductions drawn are far reaching. From the fact that the strain of herpes virus employed was immediately suppressed in the brain of the guinea pig, it is concluded that similar happenings take place in human cases of epidemic encephalitis. In this simple way does Dmitrieff dispose of the innumerable failures to infect rabbits with material taken from cases of epidemic encephalitis. To account for the herpes virus origin of the latter disease, he adopts what is essentially Levaditi's² view of special predisposing conditions of the nervous organs coinciding with particularly neurotropic strains of the virus, and he finds in epidemic influenza a possible source of the predisposing agent.

Rose and Walthard's study covered a wider field. So far as their paper is concerned, we need take into account only those parts which bear on our theme, namely the extent to which the reaction of the central nervous system of the guinea pig to the presence of herpes virus may be used as a guide and measure of what goes on in human cases of epidemic encephalitis. The experiments of Rose and Walthard have also, as will appear, a somewhat wider significance, since their inadequacy can be easily traced to the use for the tests of strains of herpes virus not of high but only of moderate virulence. The more deeply the problem of herpes virus is probed, the more clearly is it discerned that original virulence is not only a highly variable quantity,

⁷ Dmitrieff, S., *Z. Hyg. u. Infektionskrankh.*, 1926, cvi, 547.

but also that strains are to be selected according to this original grade of virulence for the rabbit, and as suitable for the particular experimental tests which it is proposed to carry out. Had Dmitrieff and Rose and Walthard employed highly virulent strains, their results and conclusions could not but have been very different.

Rose and Walthard sum up their experiments in the following words:

Rabbits inoculated subdurally with the guinea pig brain may succumb to typical encephalitis. Corneal and pad inoculations give poor results. In many instances, however, the subdural inoculations fail. The similarity, therefore, to what happens in von Economo's encephalitis is very close. Should this observation be confirmed, we shall have before us a model, supplied by the use of the guinea pig for inoculation, with which to explain what happens with autopsy material taken from human cases of encephalitis, in which the herpes virus is so rarely demonstrable. While of course this model does not suffice to establish the herpes virus etiology of von Economo's encephalitis, yet the observation does remove one of the most important objections to the assumption of this etiological relationship.

This point of view is upheld by Doerr,³ in whose laboratory the experiments were made, and by Rose and Walthard. It is, however, based not on many and varied, but only upon a few experiments, of which the following is an instance.

A guinea pig was given a pad inoculation of rabbit brain herpes virus. On the 5th day, paralysis of the extremities appeared, and on the 6th day the animal was killed. The lumbar spinal cord was inoculated into two guinea pigs: in one into the brain and pad, and in the other into the pad and cornea. A control rabbit received a subdural and corneal inoculation. The control developed keratoconjunctivitis and encephalitis, and succumbed on the 5th day.

Now, the guinea pig receiving pad and corneal inoculations responded with both keratoconjunctivitis and paralysis of the hind quarters, from both of which symptoms it recovered. The guinea pig receiving subdural and pad inoculations, responded with paralysis of the hind quarters without showing, however, any signs of encephalitis.

The result is interpreted as showing the extraordinary defense to and capacity for localizing the herpes virus possessed by the central nervous tissues of the guinea pig. A strain of herpes virus capable of passing into and injuring the spinal cord, from a skin surface (so called spontaneous neurotropy),⁸ is incapable of attacking the brain on direct injection. Doerr's comment on the experiments is to the effect that many strains of herpes virus are active when inoculated into the skin and cornea, and ineffective when introduced into the brain of guinea pigs. He remarks that this extraordinary variability of the virus is a factor to be reckoned with in considering the etiology of von Economo's encephalitis. In an addendum to his paper, Dmitrieff cites the experiments of Rose and Walthard as upholding his contentions.

⁸ Rose, G., *Centr. Bakt., 1. Abt., Orig.*, 1925-26, xcvi, suppl., 146.

This point of view, that the guinea pig occupies an independent and strategic position in respect to the extraordinary resistance to infection displayed by the brain tissue, is presented by Rose and Walthard from another point of view, namely that of the effects of corneal inoculations in producing symptoms of brain involvement. It will be recalled that Doerr and Vöchting¹ first produced herpes virus keratoconjunctivitis in guinea pigs. Because of the smaller size of the eye, the inflammation is less impressive in guinea pigs. Rose and Walthard are correct in stating that the pronounced opacity of the cornea is a regular symptom of the keratitis in guinea pigs, and only an occasional symptom in rabbits. They state also that they never observed general symptoms (encephalitis) to follow corneal inoculation, although the same strain of virus injected into the pad produces myelitis. They record a single exception to the rule, in which a guinea pig with double corneal inoculation showed salivation for 2 days and later completely recovered from these general effects, as well as from the eye lesions.

The experimental results described by Dmitrieff and by Rose and Walthard are in themselves of interest. The extent of their importance depends, however, upon their general applicability. Unless indeed they are generally applicable, it may be questioned whether their bearing on the disputed question of the etiology of epidemic encephalitis, so called von Economo's encephalitis, is as significant as they suppose. We already know that the highly susceptible rabbit suffices to distinguish differences among strains of herpes virus.⁹ May not the less susceptible guinea pig merely act to separate the strains more sharply, by totally suppressing the weak, partly suppressing the medium, and not suppressing at all the strong strains? The experiments which follow would seem to support this interpretation of the reaction of guinea pigs to intracerebral and corneal inoculations of strains of herpes virus.

The experiments to be described have been alluded to in an earlier publication.¹⁰ Moreover, in that publication a protocol is given which shows that a strong virus, implanted on the cornea of the guinea pig, may ascend to the brain and produce fatal encephalitis.

EXPERIMENTAL.

We possess in the H.F. I and Beckley specimens two highly virulent strains of herpes virus. Thus far, these strains have been studied

⁹ Flexner, S., and Amos, H. L., *J. Exp. Med.*, 1925, xli, 233.

¹⁰ Flexner, S., *J. Gen. Physiol.*, 1925-27, viii, 713.

chiefly in rabbits. Yet as early as 1922, a number of inoculations into guinea pigs were made, and it was found that brain to brain passages took place. Since this is the class of experiment which was carried out more systematically later only sample protocols of the earlier tests will be given here.

Guinea Pigs.—Nov. 20, 1922. Two, 250 gm. guinea pigs, numbered 1 and 2, were inoculated intracerebrally¹¹ with fresh rabbit brain virus, H.F. I. Guinea Pig 1 showed no striking symptoms, and died on Nov. 26 (brain transferred to Guinea Pigs 3 and 4). Guinea Pig 2 was slow and tremulous on Nov. 27; salivating and convulsive on Nov. 28, on which date death occurred (brain injected into Guinea Pigs 5 and 6).

Nov. 28, 1922. Two, 300 gm. guinea pigs, numbered 3 and 4, received intracerebral injection of brain from Guinea Pig 1. Dec. 1. Guinea Pigs 3 and 4 salivating. Dec. 2. Both guinea pigs convulsive; died on this date.

Nov. 28, 1922. Two guinea pigs, numbered 5 and 6, inoculated intracerebrally with brain from Guinea Pig 2. Dec. 4. Guinea Pig 5 circles to left (side of inoculation) and is tremulous. Guinea Pig 5 died on Dec. 5. Guinea Pig 6 died without showing symptoms.

Further passages were not made. The histology was characteristic of virus encephalitis.

Jan. 23, 1923. Two guinea pigs, 300 gm. each, numbered 9 and 10, received intracerebral injections of fresh rabbit brain, Beckley strain. Guinea Pig 9, Jan. 28, tremulous, ataxic, gnashing. Jan. 29. Salivation. Jan. 30. Death. Guinea Pig 10 circled on Jan. 27 to side of inoculation and died on Jan. 28.

No further passages were made at this time.

Although this series of tests is small and incomplete, it nevertheless brings out two facts: first, that an active virus produces fatal encephalitis in the guinea pig, attended sometimes by symptoms similar to those of the rabbit, and sometimes progressing asymptotically; and second, that passage of the virus from guinea pig to guinea pig by cerebral inoculation is possible. Note should be given to the demonstration that the brain of an inoculated guinea pig which succumbs without showing symptoms produces on cerebral inoculation of other guinea pigs, encephalitis accompanied by typical symptoms.

Serial Passage of H.F. I Virus.

The repetition of the guinea pig inoculations of the H.F. I virus, undertaken after the papers of Dmitrieff and Rose and Walthard

¹¹ All operations were performed under ether anesthesia.

appeared, covered a larger number of passages, both by the cerebral and by the corneal routes.

Cerebral Inoculations.

Guinea Pigs.—1st passage. Oct. 25, 1926. Two guinea pigs, A and B, 300 gm. each, inoculated intracerebrally with fresh rabbit brain virus H.F. I. Guinea Pig A developed fever ($41.1^{\circ}\text{C}.$), tremor, salivation, and weakness of legs; died Nov. 1. Guinea Pig B also developed fever and tremor; died Oct. 29.

2nd passage. Oct. 29. Two guinea pigs, C and D, inoculated intracerebrally with fresh brain, Guinea Pig B. Guinea Pig C developed fever and tremor; died Nov. 4. Guinea Pig D developed, in addition, salivation and convulsions; died Nov. 6.

3rd passage. Nov. 4. Two guinea pigs, E and F, injected intracerebrally with fresh brain of Guinea Pig C. Both showed encephalitic symptoms, including fever ($41.2^{\circ}\text{C}.$); died Nov. 10 and 12 respectively. The animals which survive longest tend to develop the more striking symptoms.

4th passage. Nov. 12. Two guinea pigs given intracerebrally injection of fresh brain of Guinea Pig E. One died of trauma. The other, Guinea Pig G, showed tremor and salivation; died Nov. 22. Two additional guinea pigs, H and I, were injected intracerebrally with fresh brain of Guinea Pig F on Nov. 10. Both developed symptoms consisting of fever ($41.3^{\circ}\text{C}.$), tremor, convulsions, and salivation; died Nov. 18 and 19 respectively.

5th passage. Nov. 18. Two guinea pigs, J and K, inoculated intracerebrally with fresh brain of Guinea Pig I. Both developed symptoms of encephalitis and died Nov. 25 and 26 respectively.

6th passage. Nov. 26. Four guinea pigs were inoculated, two with fresh brain of Guinea Pig J, and two with fresh brain of Guinea Pig K. One of the four died of trauma; the other three (Guinea Pigs L, M, and N) showed typical symptoms of encephalitis; died on Dec. 4 and 5.

7th passage. Dec. 6. Four guinea pigs were injected intracerebrally with fresh brain of Guinea Pigs L and M of previous passage. All (Guinea Pigs O, P, Q, R) developed characteristic symptoms; died Dec. 12 and 13.

8th passage. Dec. 14. Two guinea pigs (S and T) inoculated intracerebrally with fresh brain of Guinea Pig R, 7th passage. Both developed symptoms; died Dec. 20.

9th passage. Dec. 21. Two guinea pigs (U and V) injected intracerebrally from Guinea Pig S of 8th passage. Both developed symptoms. One (U) died Dec. 27; the other (V) recovered.

10th passage. Dec. 28. Two guinea pigs (W and X) inoculated intracerebrally with fresh brain of Guinea Pig U, 9th passage. Both guinea pigs developed symptoms; died Jan. 3, 1927.

At this point, the series of inoculations was interrupted. The results of the series were established by histological study of the brains of the guinea pigs and by return inoculations intracerebrally and corneally into rabbits at the second and third guinea pig passages. Five rabbits receiving inoculations of guinea pig brains, either subdurally or corneally, developed encephalitis and succumbed. The fact should be stressed that virus encephalitis in the guinea pig sometimes runs an asymptomatic course to a fatal issue. The brains of such animals produce symptomatic encephalitis on passage inoculation. This condition was observed in the 1922 and again in the 1926 virus passages.

Corneal Inoculations.

The series of corneal inoculations of guinea pigs with H.F. I virus assumes importance by reason of the fact that Rose and Walthard⁵ and Doerr³ emphasize the point that no strain of herpes virus has been described which passes from the eye to the brain, inducing fatal encephalitis. The H.F. I virus is capable of making this passage and of causing death.

Guinea Pigs.—Guinea Pig I. Nov. 4, 1926. Right eye cocaineized and cornea scarified with cataract knife dipped in 10 per cent suspension of fresh brain of Guinea Pig C, 2nd cerebral passage. Severe keratoconjunctivitis followed, attended by fever (41.2°C.), and on 6th day by symptoms of brain involvement: circling to right, tremor, ataxia, salivation. Death on Nov. 16, or 12 days after inoculation.

Guinea Pig II.¹² Nov. 8. Right eye inoculation as before with exudate from Guinea Pig I. First keratoconjunctivitis, then encephalitis developed, leading to death on Nov. 18, or 10th day after inoculation.

Further inoculations were made with the fresh brains of Guinea Pigs I and II. Material from Guinea Pig I was introduced into the cornea of one guinea pig and the brain of two other guinea pigs, without effect. The conclusion is that the virus was no longer active on the 12th day. The brain of Guinea Pig II was injected intracerebrally into two guinea pigs, of which one succumbed to virus encephalitis on the 6th, and the other on the 12th day. Another guinea pig which received a corneal inoculation of the brain, developed keratoconjunctivitis attended by salivation, from which recovery took place.

¹² This is the animal whose protocol is given in "Epidemic encephalitis and simple herpes," Flexner, S., *J. Gen. Physiol.*, 1925-27, viii, 713.

The power to pass from the cornea to the brain possessed by even strong strains of herpes virus is determined not by the virus alone, but is conditioned by the individual guinea pig subjected to inoculation. Thus of four guinea pigs which were given corneal inoculations of fresh H.F. I guinea pig brain virus, one at the third and three at the sixth guinea pig passage, all four developed keratoconjunctivitis, but one only exhibited encephalitic symptoms to which it succumbed. The protocol of this animal follows.

Guinea Pig.—Dec. 4, 1926. Cocainized cornea scarified and inoculated with H.F. I virus, 6th guinea pig brain passage. Typical keratoconjunctivitis. Dec. 11. Salivation, tremor, falling. Dec. 13. Death.

There remains to be described one other type of corneal inoculation with strong herpes virus, namely that in which following the keratoconjunctivitis the guinea pigs develop encephalitic symptoms from which they recover. The next two protocols illustrate this result.

Guinea Pig.—Oct. 25, 1926. Right eye cocainized and scarified with cataract knife dipped in suspension of fresh rabbit brain virus H.F. I. Typical keratoconjunctivitis appeared. On 6th day the temperature rose to 41.2°C.; on 10th day, tremor, ataxia, and circling to right were noticed. These symptoms and the inflammation of the eye gradually subsided, recovery becoming complete.

Guinea Pig.—Oct. 29, 1926. Right eye inoculation as before with fresh guinea pig brain virus H.F. I, 2nd brain passage. Moderately severe keratoconjunctivitis. On 10th day, tremor and salivation, and temperature of 40.7°C. During the next few days the eye inflammation subsided and the nervous symptoms disappeared.

We shall now describe the inoculation of guinea pigs with the Beckley and Levaditi strains of herpes virus. These two strains have been studied by us in rabbit passages and found to be weaker than H.F. I.⁹ Moreover, they differ from each other, as the Levaditi strain is weaker than the Beckley. As tested on the rabbit, the order of virulence is H.F. I, Beckley, and Levaditi.

Beckley Virus Series.

Two separate tests were made with the Beckley virus injected intracerebrally into guinea pigs. The two sets are not in precise agreement, since the fatalities in one exceeded those in the other test. But they are in agreement in bringing out the fact that the Beckley is less active than the H.F. I strain.

Cerebral Inoculations.—

Test I. Guinea Pigs A and B.—Dec. 31, 1926. Injected into right cerebrum suspension of fresh rabbit brain Beckley virus. Fever ($41.1^{\circ}\text{C}.$), tremor, salivation, and paralysis of extremities developed. Death of Guinea Pig A occurred on Jan. 8, 1927. Guinea Pig B was killed on Jan. 6.

Guinea Pigs C and D.—Jan. 7, 1927. Inoculated as in preceding test with fresh brain virus of Guinea Pig B. Symptoms of encephalitis appeared in both guinea pigs and death followed on Jan. 14.

Guinea Pigs E and F.—Jan. 17, 1927. Injected as in preceding, with fresh brain virus of Guinea Pig D. Symptoms of encephalitis developed and Guinea Pig E died on Jan. 24; Guinea Pig F died on Jan. 22.

Guinea Pig G.—Jan. 25, 1927. Inoculated as before with fresh brain virus of Guinea Pig E. Developed symptoms of encephalitis, including tremor, gnashing, convulsions, salivation, and impaired eyesight; death on Feb. 2.

Guinea Pig H.—Feb. 4, 1927. Inoculated as before with fresh brain virus of Guinea Pig G. No symptoms appeared and animal remained well.

Guinea Pigs I and J.—Feb. 7, 1927. Injected intracerebrally with virus from Guinea Pig G, glycerolated for 5 days. No effect was produced.

The sudden loss of activity on the part of the Beckley strain of virus at the fifth passage in guinea pigs led us to repeat the test. The series of protocols follows.

Test II. Guinea Pigs A and B.—Feb. 21, 1927. Intracerebral inoculation of fresh rabbit virus, Beckley strain. Symptoms of severe encephalitis developed, so that the moribund animals were killed on Feb. 28 and Mar. 3 respectively.

Guinea Pigs C and D.—Mar. 1, 1927. Cerebral inoculation from Guinea Pig B. Symptoms of encephalitis, including tremor, salivation, and convulsions, appeared. Guinea Pig C died on Mar. 7; Guinea Pig D was killed on Mar. 5.

Guinea Pigs E and F.—Mar. 5, 1927. Cerebral inoculations from Guinea Pig D. Guinea Pig E showed slight, fleeting symptoms only and recovered; Guinea Pig F showed more pronounced, but not severe symptoms, and was killed on Mar. 11.

Guinea Pigs G and H.—Mar. 11, 1927. Cerebral inoculation with fresh brain of Guinea Pig F. No marked symptoms arose and animals remained well.

The result of the passage of the Beckley virus through the brains of guinea pigs is definite and shows that the virus, as derived from the rabbit, is active enough in the first removes to produce fatal encephalitis, but at the fourth or fifth passage suddenly loses power and fails to excite marked symptoms in the inoculated guinea pigs.

Corneal Inoculations.—The preceding tests were paralleled with two series of tests of the Beckley virus inoculated into the cornea. The first of the eye series is incomplete, but is recorded for its intrinsic

interest. The second is complete and brings out the rather sudden failure in potency of the virus in the manner of the intracerebral passages.

Test I. Guinea Pigs A and B.—Dec. 30, 1926. Cocainized right eye scarified with cataract knife dipped in 10 per cent suspension of fresh rabbit brain Beckley virus. Characteristic keratoconjunctivitis followed by fever (41.1° to 41.7°C.) on Jan. 6, 1927, coincident with appearance of tremor, salivation, and convulsions. Death of both animals on Jan. 10.

Guinea Pigs C and D.—Jan. 11, 1927. Intracerebral injections of fresh brain virus of Guinea Pig A. Typical symptoms of encephalitis developed; death on Jan. 18 and 19 respectively.

Guinea Pigs E and F.—Jan. 10, 1927. Corneal inoculations with fresh brain of Guinea Pig A. Keratoconjunctivitis followed by encephalitis appeared; death of Guinea Pig E on Jan. 17, of Guinea Pig F on Feb. 4.

Test II. Guinea Pigs A and B.—Jan. 6, 1927. Corneal inoculations with fresh brain of guinea pig of 1st Beckley strain passage. Keratoconjunctivitis and encephalitis; death on Jan. 17 and 21 respectively.

Guinea Pigs C and D.—Jan. 14, 1927. Corneal inoculation with fresh brain of guinea pig of 2nd Beckley strain passage. Keratoconjunctivitis and encephalitis; death of both animals on Jan. 29.

Guinea Pigs E and F.—Jan. 24, 1927. Corneal inoculation with fresh brain of guinea pig of 3rd Beckley strain passage. Keratoconjunctivitis and encephalitis; death of Guinea Pig E on Feb. 4, and sacrifice of F on Feb. 2.

Guinea Pigs G and H.—Feb. 2, 1927. Corneal inoculation with fresh brain of guinea pig of 4th Beckley strain passage. Severe keratoconjunctivitis without symptoms of encephalitis followed by recovery.

Guinea Pigs I and J.—Mar. 11, 1927. Corneal inoculation with fresh brain of guinea pig of 3rd Beckley strain passage. The cornea of one, but not of the other guinea pig became inflamed, and no cerebral symptoms appeared.

Levaditi Virus Series.

The experiments so far described establish the guinea pig as selective for strains of herpes virus of differing potencies as is the rabbit, the distinction being that because of greater natural resistance to infection the guinea pigs make a sharper distinction of virulence than do rabbits. Hence we should expect that a strain of the virus relatively weak for the rabbit should prove even less effective for the guinea pig than either the H.F. I or the Beckley strain. Levaditi Strain 6 is, according to Flexner and Amoss,⁹ such a weak strain. It has, therefore, been inoculated into guinea pigs by way of the brain and the cornea, and has

behaved in conformity with its rabbit propensities. Of the three strains studied by us in guinea pigs, it alone corresponds in its action to the descriptions given by Dmitrieff and by Rose and Walthard. The protocols of the experiments follow in brief.

Test I. Guinea Pigs I and II.—Jan. 26, 1927. Cerebral inoculation with fresh rabbit brain Levaditi virus. Guinea Pig I showed no symptoms and remained well. Guinea Pig II developed fever (40.8°C.) and tremor on the 3rd day. No accentuation of the symptoms but emaciation set in. Killed on 13th day. The brain of this animal injected intracerebrally into two guinea pigs, III and IV, and into a rabbit was without result.

The original rabbit brain virus inoculated into the cornea of two guinea pigs produced keratoconjunctivitis, unattended by cerebral symptoms from which recovery ensued.

Test II. Guinea Pigs V and VI.—Feb. 15, 1927. Cerebral injection of fresh rabbit brain Levaditi virus. No symptoms followed and the animals remained well.

Test III. Two Guinea Pigs.—Feb. 22, 1927. Injected as in previous tests with fresh rabbit brain Levaditi virus without producing symptoms.

Test IV. Two Guinea Pigs.—Mar. 23, 1927. Received fresh rabbit brain Levaditi virus by cerebral injection, again without result.

The Levaditi virus employed for the inoculations was active for the rabbit on intracerebral injection and was used on the 6th day of the encephalitis for injecting the guinea pigs.

As the protocols show, only the first cerebral inoculation was positive and that in the first test only. In this test, the attempted second passage to guinea pigs failed. Corneal inoculation succeeded in the first test, but no evidence of extension of the inflammatory process to the brain was detected.

DISCUSSION.

The experiments described in this paper leave no doubt that the guinea pig is only less subject to infection with the herpes virus than is the rabbit. The inoculations of herpes virus in the guinea pig in the past have covered too narrow and restricted a variety of virus strains. There is no doubt that the strain employed by Dmitrieff was a weak one, and the one employed by Rose and Walthard little, if any, stronger. Flexner and Amoss⁹ had already placed the Doerr strain—presumably the one used by Rose and Walthard—among the weak strains, on the basis of rabbit tests and of reaction to glycerolation.

The Levaditi strain had also been classed as a weak strain; and the tests on guinea pigs bring these three strains—Dmitrieff, Rose and Walthard, and Levaditi—in the same category of strength or virulence. The fact should be emphasized that so weak a virus as the Doerr strain, inoculated into the skin of guinea pigs, proved to be active enough to pass regularly from it to the spinal cord, thus displaying the property called spontaneous neurotropy by Rose.⁵

The H.F. I and Beckley strains of virus possess far greater virulence for guinea pigs as well as for rabbits. Their strength has not notably diminished during the 5 and 4 years respectively since their original isolation and after very many rabbit passages. Both are capable of being passed from guinea pig to guinea pig by means of cerebral and corneal inoculations, although the H.F. I strain, the stronger by rabbit test, alone seems capable of indefinite passage. Both also are capable of passing from the cornea to the brain, inducing fatal encephalitis. Hence they belong in a totally different category from the three other strains already mentioned.

The theoretical importance of the manner of response of guinea pigs to herpes virus inoculation arises from the use which has been made of fragmentary knowledge to support the hypothesis of the virus etiology of epidemic encephalitis. The paradox of this ubiquitous virus, ordinarily so readily implanted upon the rabbit and yet so rarely found in cases of epidemic encephalitis, has effectually barred the way to the wider adoption of the hypothesis. Hence the eagerness with which its upholders seized upon the guinea pig experiments as supporting their belief. The experiments given in this paper make it more than doubtful whether the support is valid and whether, indeed, any light whatever has been thrown on the etiology of epidemic encephalitis through the tests made on guinea pigs. Rather it would seem that the nature of the inciting agent of that disease remains still a problem for future solution.

SUMMARY.

The guinea pig is subject to cerebral and corneal inoculation of the herpes virus.

The effects of the inoculations vary with the strength or degree of virulence of the virus.

Weak strains of the virus are implanted on the cerebrum with difficulty and strong strains with ease.

Weak strains are quickly suppressed by the brain and strong strains may be passed indefinitely from brain to brain of the guinea pig. Strains of intermediate potency can be passed for a limited number of times only.

Weak strains induce keratoconjunctivitis without brain involvement, while strong strains invade the brain from the eye and produce fatal encephalitis. In the latter case, the brain contains active virus inoculable upon the cornea and into the brain of rabbits and guinea pigs. Strains of intermediate potency produce keratoconjunctivitis accompanied by mild symptoms of encephalitis, from which recovery results.

The guinea pig serves even more definitely than the rabbit to distinguish grades of virus according to strength or virulence. There is no difference of kind but only of degree of response to inoculation of herpes virus in the rabbit and the guinea pig.

The etiology of epidemic encephalitis has not, therefore, been brought appreciably nearer solution by experiments with herpes virus carried out in guinea pigs.

I wish to thank Mr. Peter Haselbauer for very valuable assistance in the carrying out of the experiments on which the two present papers are based.

ULTRA-VIOLET LIGHT AND VACCINE VIRUS.

I. THE REACTION OF IRRADIATED SKIN TO VACCINE VIRUS.

BY THOMAS M. RIVERS, M.D., HENRY STEVENS, AND FREDERICK L. GATES, M.D.

(From the Hospital and the Laboratories of The Rockefeller Institute for Medical Research.)

PLATE 5.

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Ledingham (1) reported that the injection of India ink into the skin of rabbits induced a local refractory state to the action of vaccine virus, *i.e.*, the visible reaction usually produced by vaccine virus failed to occur in parts of the skin previously treated with ink. Carnot and his coworkers (2) determined that areas of rabbits' skin repeatedly exposed to ultra-violet light were less susceptible to the action of vaccine virus than were untreated areas of skin in the same animal. Le Fèvre de Arric observed that the shaved skin (rabbits') exposed to Roentgen rays was more resistant 14 days later to vaccine virus than was untreated skin (3). The observations of these men and a desire to obtain information concerning the modification of the picture of vaccinia in rabbits by means of non-specific procedures led to the experiments which are reported in the present paper.

Methods and Materials.

Vaccine Virus.—The vaccine virus used in these experiments was obtained originally from Dr. Noguchi. It is free from ordinary bacteria and is propagated in the testicles of rabbits (4). A large amount of the virus was prepared and glycerolized. To facilitate comparison of results, the same virus emulsion was used in all experiments.

Animals.—The majority of the rabbits used were albinos. Other kinds of rabbits were employed, however, and the results observed in them were similar to those obtained in albinos.

Irradiation and Vaccination.—The hair was removed from large areas of skin by shaving. In the animals that received only one irradiation, selected areas of

the shaved skin were exposed to the direct rays of the quartz mercury arc (60 to 65 volts D. C.) at a distance of 50 cm. for periods of time ranging from 5 to 40 minutes. The control areas of skin were protected against the action of the ultra-violet light by screens of lead-foil. At different intervals of time following the completion of the irradiation the treated and untreated areas of skin were scarified and inoculated with vaccine virus. In the rabbits that received repeated irradiations, shaved areas of skin over the dorsal surface of the animals were exposed to the action of ultra-violet light at a distance of 1 meter for 15 to 30 minutes, at daily intervals for 1 or 2 weeks. After the repeated exposures to the light, the treated skin was scarified and inoculated with vaccine virus. Control areas of skin of the same animals, on the lateral surface of the abdomen and thorax, were shaved and inoculated in a manner similar to that employed in the treated areas. As an additional control, the shaved skin over the dorsal surface of rabbits not treated with ultra-violet light was also scarified and inoculated with vaccine virus.

In studying the effect that ultra-violet light has upon the skin of rabbits in regard to the activity of vaccine virus subsequently inoculated upon it, the work was divided into two parts; the first dealt with the effect of single exposures, the second with repeated irradiations.

Effect of Single Irradiations.

A number of rabbits were exposed to the rays of the quartz mercury arc for 5, 10, or 30 minutes. 30 to 60 minutes after the exposures to the light were completed, areas of treated and untreated skin were scarified and inoculated in the usual manner with vaccine virus. Nine experiments were performed in the first series. The results are summarized in Table I.

The results shown in Table I indicate that the activity of vaccine virus is delayed and mitigated (see Fig. 1) when the virus is inoculated on the recently irradiated skin of a rabbit. Furthermore, the lesions that develop in treated skin are frequently more deeply situated than are those in untreated skin.

The fact that the lesions in the irradiated skin were delayed and more deeply situated than usual led us to suspect that they might have resulted from virus transported to the treated skin through the blood stream from the lesions in the untreated skin. Experiments were performed to obtain information concerning the source of the virus producing these deep lesions.

An area of a rabbit's skin was irradiated for 40 minutes in the usual manner. Thirty minutes later the irradiated skin was gently scarified and inoculated with vaccine virus. Care was taken not to soil the untreated skin with virus. Another rabbit whose skin had not been exposed to ultra-violet light was inoculated in the usual manner. The lesions in the control rabbit appeared on the 3rd day, and

TABLE I.

The Reaction of Irradiated Skin to Vaccine Virus Inoculated upon It Shortly after Single Irradiations.

Rabbit No.	Distance from arc	Time of exposure	Voltage of D.C.	Results of vaccination	
				Irradiated skin	Untreated skin
1	50	30	60-65	One pustule appeared 6th day	Eleven pustules appeared 3rd day
2	"	"	"	No lesions	Numerous lesions
3	"	"	"	+	+++
4	"	"	"	First definite sign 6th day	Appeared 3rd day
5	"	"	"	+	++++
6	"	"	"	First definite sign 6th day	Appeared 3rd day
7	"	"	"	+	++++
8	"	"	"	First definite sign 6th day	Appeared 3rd day
9	"	"	"	?	++
10	"	"	"	Doubtful take 6th day	Appeared 3rd day
11	"	"	"	?	+++
12	"	"	"	No definite lesions	Appeared 3rd day
13	"	5	"	++	+++
14	"	10	"	Appeared 4th day	Appeared 3rd day
15	"	5	"	+	+++
16	"	10	"	Appeared 4th day	Appeared 3rd day
17	"	5	"	++	+++
18	"	10	"	Appeared 4th day	Appeared 3rd day
19	"	5	"	+	+++
20	"	10	"	Appeared 4th day	Appeared 3rd day

+ indicates a positive reaction to vaccine virus. The number of + signs indicates the severity of the reaction.

? indicates the presence of a doubtful reaction.

were confluent. Only a few lesions developed in the treated rabbit; they appeared on the 5th day and were deeply situated in the skin. Several experiments of this character were performed and the results were always the same, provided a potent virus was used.

Two rabbits were shaved on both sides of the thorax and abdomen. Two areas on one side of each rabbit were irradiated for 20 and 40 minutes respectively.

Strips of skin for controls were protected from the effect of the light. Immediately after irradiation the untreated side of each rabbit was inoculated with vaccine virus in the usual manner. The lesions at the sites of inoculation appeared on the 3rd day, and later coalesced. On the 5th day a few discrete lesions were observed in the irradiated skin. None were seen, however, in the nearby skin protected by foil.

The results of the two sets of experiments just described indicate that the deep, delayed lesions in the irradiated skin of the rabbits employed for the experiments summarized in Table I may have resulted either from the virus inoculated directly on the treated skin or from virus transported to it by the blood from lesions in the untreated skin.

It seemed of interest to ascertain whether irradiated skin possesses virucidal properties for vaccine virus. This matter was investigated in the following manner.

The skin on one side of a rabbit was irradiated for 40 minutes. Immediately after the completion of the irradiation the rabbit was sacrificed. The superficial layers of the treated skin were then removed and emulsified. As a control, skin from the untreated side was removed and emulsified in a similar manner. Portions of each emulsion were mixed with small amounts of vaccine virus and incubated at 37°C. for 1 hour. These mixtures were then smeared on areas of scarified skin of the same rabbit. Lesions developed at all points where the mixtures were placed.

From the above experiment it may be concluded that irradiated skin is not virucidal for vaccine virus and that the results summarized in Table I were not dependent upon a factor of this nature.

When it had been determined that the reaction to vaccine virus was delayed and mitigated in irradiated skin inoculated 30 to 60 minutes after treatment, experiments were performed to ascertain the type of reaction that occurs in skin inoculated with vaccine virus 18 to 72 hours after irradiation.

A number of rabbits were shaved and areas of skin were irradiated for 5, 10, or 30 minutes. Areas of skin for control on each rabbit were protected by foil. Areas of treated and untreated skin were inoculated with vaccine virus after varying intervals of time had elapsed following irradiation. At the time of inoculation the treated skin was usually red and edematous. The results of the experiments are summarized in Table II.

From the results of experiments summarized in Table II it appears that skin irradiated for a short time 24 to 72 hours prior to inoculation

TABLE II.

The Reaction of Irradiated Skin to Vaccine Virus Inoculated upon It 18 to 72 Hours after Single Irradiations.

Rabbit No.	Distance from arc	Time of exposure	Voltage of D.C.	Lapse of time between irradiation and inoculation	Results of vaccination	
					Irradiated skin	Untreated skin
	<i>cm.</i>	<i>min.</i>		<i>hrs.</i>		
10	50	30	60-65	24	+++ Appeared 4th day	+ Appeared 4th day
11	"	"	"	"	+++++ Appeared 3rd day*	+++ Appeared 2nd day
12	"	"	"	"	+++++	+++
13	"	"	"	"	Appeared 3rd day +++++	Appeared 2nd day +++
14	"	"	"	18	Appeared 3rd day Slow in developing =	Appeared 2nd day +++
15	"	"	"	"	Interpretation difficult =	Appeared 3rd day +++
16	"	5	"	48	Interpretation difficult +++++	Appeared 3rd day +++++
	"	10	"	"	Appeared 3rd day +++++	Appeared 3rd day +++++
17	"	5	"	72	Appeared 3rd day +++++	Appeared 3rd day +++++
	"	10	"	"	Appeared 2nd day +++++	Appeared 2nd day +++++
					Appeared 2nd day	Appeared 2nd day

* Frequently difficult to determine the time of appearance of the lesions in the irradiated skin, inasmuch as the skin was red and edematous as a result of the irradiation.

+ indicates a positive reaction to vaccine virus. The number of + signs indicates the severity of the reaction.

= indicates a positive reaction masked by the irritation of the skin produced by the light.

reacts more violently to vaccine virus than does untreated skin in the same animal (see Fig. 2).

Effect of Repeated Irradiations.

The effect of single exposures to ultra-violet light upon the types of reaction produced by vaccine virus subsequently inoculated on the treated skin aroused interest in the question as to what effect repeated irradiations would have upon such reactions. The matter was investigated in the following manner.

A number of rabbits were shaved. Certain areas of skin were irradiated for 15 or 30 minutes daily for about a fortnight. At first the skin became red after each irradiation, and after a few treatments desquamation of the superficial layers

TABLE III.

Effect of Repeated Irradiation upon the Skin in Respect to Its Reaction to Vaccine Virus.

Rabbit No.	Distance from arc	Voltage of D.C.	Time of each exposure	No. of exposures	Lapse of time between last irradiation and inoculation	Results of vaccination		
						Irradiated skin	Untreated skin	Skin of untreated animal
18	m.	60-65	min.		hrs.			
18	1	60-65	30	9 in 11 days	1	++	No test	++++
19	"	"	"	11 " 13 "	"	+	+++	++++
20	"	"	15	12 " 14 "	"	++	++++	No test
21	"	"	"	11 " 12 "	48	±	++++	" "

+ indicates a positive reaction to vaccine virus.

± indicates a weakly positive reaction to vaccine virus. The number of + signs indicates the severity of the reaction.

of the epidermis occurred. Subsequent to this no macroscopic changes were noted in the skin, following the daily irradiations. Finally the treated skin was inoculated with vaccine virus 1 hour or 48 hours after the last exposure. Areas of untreated skin in the same animals and the skin of untreated rabbits were also inoculated with virus as controls. At the time of inoculation the irradiated skin appeared to be perfectly normal. The results of the experiments are shown in Table III.

From the results of the experiments summarized in Table III, one may conclude that areas of skin repeatedly irradiated are less susceptible to the action of vaccine virus than are untreated areas of skin in the same animals.

DISCUSSION.

Rabbits' skin irradiated for a few minutes and then immediately inoculated with vaccine virus is less susceptible to the action of the virus than is untreated skin. If 24, 48, or 72 hours elapse between the time of irradiation and inoculation, however, the treated skin appears to be more susceptible than untreated skin. Teague and Goodpasture (5) have demonstrated that herpes virus produces an especially pronounced reaction in a guinea pig's skin that shows active epithelial hyperplasia produced by applications of coal tar. Cobbett and Mel-some (6), working with hemolytic streptococci, noted that rabbits' ears irritated with mustard oil were more susceptible to streptococcal infections than were control ears during the first 48 hours following the irritation, after which they became more resistant than the controls,—a finding the converse of that in our irradiated rabbits.

An explanation of these phenomena and of that which we have dealt is not readily found. Certain filtrable viruses (7), among which are vaccine virus and herpes virus, have not been cultivated in lifeless media. These active agents, however, do multiply in the presence of suitable living host cells. Furthermore, the multiplication seems to occur best in the presence of young, actively growing cells. These facts may aid in explaining the results of some of the experiments reported in the present paper. When irradiated skin was immediately inoculated with vaccine virus, the virus was placed on damaged or dead tissue,—for in the end the skin desquamated. One may presume that the reaction to the virus was delayed or mitigated because of the condition of the cells with which it came in contact. If 24, 48, or 72 hours elapsed between the time of irradiation and inoculation, the virus was placed on a skin the surviving cells of which were already stimulated toward repair while multitudes of others had come in from the blood stream. Falling on such a soil the vaccine virus may well have acted more intensely than usual. This explanation is suggested merely as a possibility.

The skin that was repeatedly exposed to ultra-violet light appeared to be normal except for a light tan noted in a few instances. Yet in spite of its normal appearance, this irradiated skin was less susceptible to the action of vaccine virus than was untreated skin. No reason can at present be offered for the enhanced resistance to vaccine virus.

It is obvious that the reaction of the skin to certain viruses may be modified by means of non-specific procedures. Furthermore, since a great deal of the evidence in favor of the identity of some of the epitheliotropic viruses has been obtained by means of cross-immunity experiments conducted in the skin of human beings and animals, it is possible that factors of a non-specific nature, generally overlooked or underestimated, are responsible for part of the confusion concerning the interrelationship existing between certain of these viruses.

SUMMARY.

Rabbit skin treated for a few minutes with ultra-violet light and then inoculated at once with vaccine virus is less susceptible to the action of the virus than is untreated skin. If 24, 48, or 72 hours elapse between the time of irradiation and inoculation, the treated skin appears to be more susceptible than is untreated skin. Skin repeatedly exposed to ultra-violet light is less susceptible to the action of vaccine virus than is non-irradiated skin.

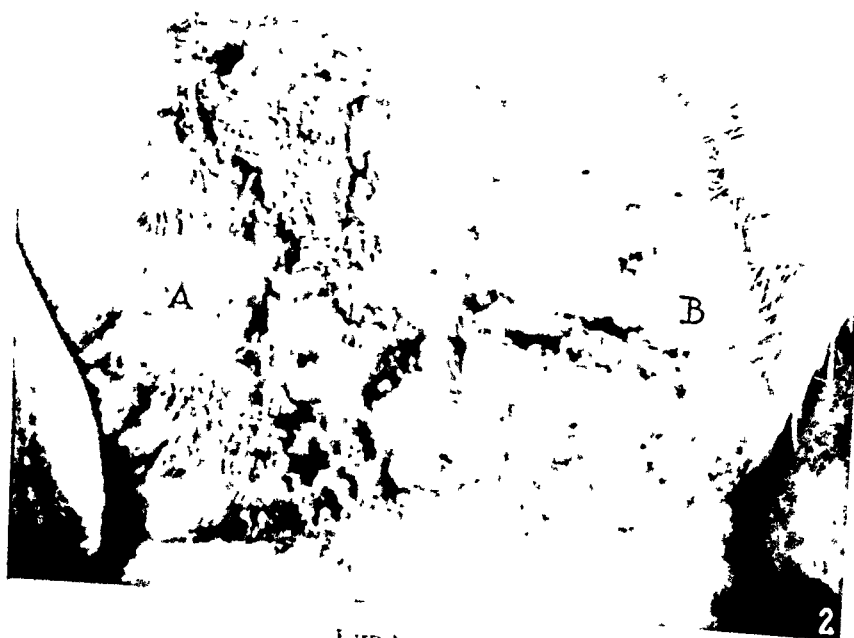
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EXPLANATION OF PLATE 5.

FIG. 1. Rabbit 1961. *A* irradiated, *B* control. Both *A* and *B* were inoculated with vaccine virus within a few minutes after irradiation of *A*. The broad dark lines observed in *A* are the effects of scarification and do not represent a reaction to vaccine virus.

FIG. 2. Rabbit 1960. *A* irradiated, *B* control. Both *A* and *B* were inoculated with vaccine virus 24 hours after irradiation of *A*.



ULTRA-VIOLET LIGHT AND VACCINE VIRUS.

II. THE EFFECT OF MONOCHROMATIC ULTRA-VIOLET LIGHT UPON VACCINE VIRUS.

By THOMAS M. RIVERS, M.D., AND FREDERICK L. GATES, M.D.

(From the Hospital and the Laboratories of The Rockefeller Institute for Medical Research.)

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It has been known for many years that ultra-violet light is bactericidal. Finsen and Dreyer (1), in 1903, were probably the first to show that light with short wave-lengths is virucidal also. More recently (2-7) other reports have appeared dealing with the effects of ultra-violet irradiation upon bacteria, viruses, and enzymes. For the most part, however, single wave-lengths have not been used in the investigations, nor have the workers determined or taken into consideration the amount of energy required to kill or to inactivate these agents at single wave-lengths in the active ultra-violet region. One of the present authors (Gates) is making a quantitative study of the action of ultra-violet light on certain active agents in the hope of obtaining additional information concerning their nature. In the present paper the results of the studies concerning the effects of ultra-violet irradiation upon one of these active agents, vaccine virus, will be presented.

Methods and Materials.

Vaccine Virus.—The source and preparation of the vaccine virus have been described in the preceding paper. Fresh virus, however, instead of glycerolated material, was used in the experiments to be reported in the present paper.

Method of Irradiating and Testing the Virus.—Three drops of fresh virus were allowed to spread over the surface of 2 per cent nutrient agar (pH 7.4) in a small Petri dish. After being surface-dried in a horizontal position, certain areas of these plates were exposed for various intervals of time before the exit slit of a large quartz monochromatic illuminator to the different spectral lines of a quartz mercury arc in the ultra-violet region between λ 2302 and λ 3126 Ångström units. The intensity at each wave-length (measured in ergs per sq. mm. by means of a

standardized thermopile and high sensitivity galvanometer) multiplied by the time of exposure gave the total incident energy for each exposure. For comparison, a thin suspension of *Staphylococcus aureus* was washed over the surface of nutrient agar also. After surface drying, certain areas of the plates were exposed to the same spectral lines, and to intensities similar to those used for the vaccine virus.

After exposure to the light, small sections of the agar covered with vaccine virus were removed from exposed areas and from unexposed (control) areas, were emulsified with Locke's solution, and both were injected in aliquot doses into the shaved skin of the same susceptible rabbit. The agar plates with the staphylococci were incubated, and the bactericidal effect of the ultra-violet irradiations upon the bacteria was determined by counting the number of colonies in each exposed area and comparing them with the number in unexposed areas of the same size. In this manner figures were obtained which made it possible to express the effect of the light upon the bacteria in percentage of bacteria killed. In dealing with vaccine virus, however, the methods of titration are very crude. Consequently the determinations of the amount of virus inactivated or killed by the light are only relatively accurate, for the readings only indicated a positive or negative skin reaction. Thus the end-point had to be reached each time before a reading could be relied upon, *i.e.*, the virus had to be exposed long enough so that no visible reaction occurred at the site of injection in the skin. Under these conditions, it was impossible to say whether a subinfecting amount of virus remained active after exposure to the light or whether more energy had been used than was necessary to inactivate all of the virus.

A number of experiments were performed in the manner described (1) to determine and (2) to compare the effects that monochromatic ultra-violet light has upon vaccine virus and upon staphylococci. The results are summarized in Table I and graphically presented in Chart 1. In similar bactericidal studies on *S. aureus* carried out within a few months of these experiments closer approximations to the actual amount of incident energy involved in the destruction of all the exposed organisms were obtained. For comparison these figures are included in the table under *Staphylococcus* II and the curve derived from them has been superimposed upon the cross-hatched areas of the chart.

The upper line in Chart 1 represents the amount of energy in ergs per sq. mm. at each wave-length adequate, or more than adequate, to reduce the quantity of active vaccine virus below the dose necessary to produce a visible lesion in the skin of a susceptible rabbit. The lower line connects observations obtained from experiments in which

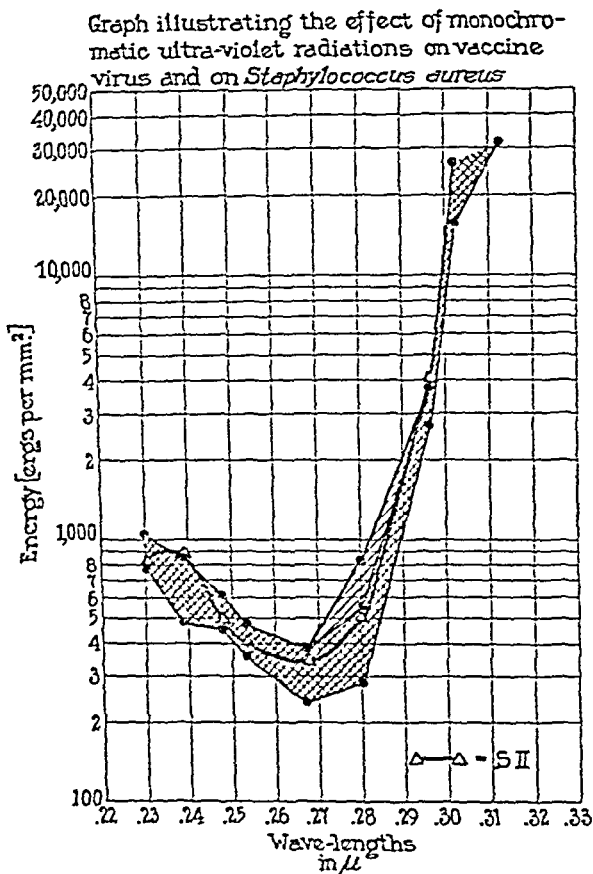


CHART 1. The incident energy required to inactivate all of a given specimen of vaccine virus lies between the upper and lower limits of the area cross-hatched // //. The energy necessary to kill all of the staphylococci falls within the area cross-hatched |||. It is evident that these areas are identical except in the case of λ 2804. At this wave-length an amount of energy that killed all of the staphylococci failed to inactivate all of the vaccine virus. Only a "lower limit" is given at λ 3126. With the amount of energy used at this wave-length neither vaccine virus nor *Staphylococcus aureus* was seriously injured. It is not known what very large amount of energy, if any, at this wave-length is injurious to the active agents studied. The curve marked S II (*Staphylococcus aureus*) represents a closer approximation of bactericidal energy as found in the experiments of another series.

the amount of energy used was not sufficient to reduce the quantity of active virus to a point below the threshold dose. In regard to staphylococci, it was found (1) that at each wave-length, except one, the amount of energy inadequate to inactivate all the virus killed only from 62 to 76 per cent of the bacteria and (2) that the energy sufficient to inactivate the virus completely also killed all of the staphylococci.

The actual curves of the bactericidal (*Staphylococcus aureus*) and the virucidal (vaccine virus) effects of ultra-violet light lie within the shaded area, which doubtless could be reduced considerably in width by further experiments. Whether the curves for vaccine virus and for

TABLE I.

Amount of Energy at Certain Wave-Lengths Sufficient to Inactivate or Kill Vaccine Virus and Staphylococcus aureus.

Wave-length	Ergs per sq. mm.		
	Vaccine virus	Staphylococcus I	Staphylococcus II
2303-30	1,040	1,040	830
2379-97	856	856	900
2482	619	619	480
2536	480	480	410
2675	356	356	345
2804	858	572	510
2967	3,708	3,708	4000
3022	26,600	26,600	—
3126	30,600 (no effect)	30,600 (no effect)	—

staphylococci are identical cannot be determined with the crude methods available at present for titrating the virus. More significant than the absolute energies involved is the general shape of the curves which might be plotted within the shaded area. A rapid decrease in the required energy between λ 3022 and λ 2804 Ångström units, a nadir at λ 2675, and a rise towards λ 2302, corresponds closely to the curve representing the absorption of ultra-violet light by protein substances, and, in general, to the curves symbolizing various reactions of protoplasm or protein derivatives of protoplasm to light of short wave-lengths. The fact that these curves are similar is interesting and suggestive. Inasmuch, however, as the vaccine virus in a testicular

emulsion is admixed with a great quantity of animal protein to which it is probably adsorbed, one should be careful not to draw final conclusions from such observations as are reported in the present paper.

SUMMARY.

Under the experimental conditions described in the present paper, it was found that the amount of energy required to kill staphylococci at single wave-lengths in the active ultra-violet region was approximately the same as that necessary to inactivate vaccine virus.

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COMPENSATORY HYPERTROPHY OF THE LUNG AFTER UNILATERAL PNEUMECTOMY.

By T. ADDIS, M.D.

*(From the Medical Department of Stanford University Medical School,
San Francisco.)*

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When one kidney or one adrenal cortex is removed the remaining organ becomes larger. This enlargement is due in the main to an increase in the volume of some or all of the cells which compose the parenchyma of the organ, and, since it follows an induced deficiency in total cell mass, the term compensatory hypertrophy has been used to designate the process. But there are some organs which do not manifest any measurable capacity for compensatory hypertrophy. The excision of one testicle is not followed by any significant change in the one which is left (1) and it has been shown that the mass of the medullary portion of the adrenal gland remains the same and the volume of the cells unaltered after unilateral adrenalectomy (2). No satisfactory explanation for this divergence in the reaction of different organs has been advanced and indeed the facts have not yet been defined with sufficient precision to warrant speculation. A quantitative comparison of the varying degrees of compensatory hypertrophy of all the different organs under identical conditions might throw light on the mechanism of the process. But in the meantime even our qualitative information is incomplete for there are no adequate data in regard to the effect of the excision of one lung.¹ The work reported here is an attempt to fill this gap in our knowledge.

¹ Three interesting and decisive studies on the effects of unilateral pneumectomy have appeared from Johns Hopkins University. Heuer and Dunn (3) described the alterations in the position of the thoracic viscera and discussed the surgical aspects of the question. The changes of the alveolar CO_2 and O_2 and in the hemoglobin concentration of the blood were determined by Heuer and Andrus (4). Andrus (5) found that the lung volume had returned to its original value in about 30 days after the operation. There was at first a marked increase in the rate of

When one lung is removed the remaining lung fills the space formerly occupied by the excised lung and pushes the heart close up against the chest wall. There is an obvious enlargement of the organ but it might be wholly due to a greater distension with air, and not at all to any increase in lung tissue. Nor did it seem that the weight of the organ would be a satisfactory measure of the amount of tissue it contained, for all the blood of the body has to pass through the remaining lung, and must add appreciably to its weight. The blood was therefore removed by perfusion with Locke's solution and the quantity of nitrogen in the washed lung was taken as the measure of the mass of lung tissue.

The left lung was removed from female albino rats on the 200th day of age under ether anesthesia. The lung was pulled up by blunt ring forceps into a long retracted intercostal incision while a catgut ligature was passed round the root of the lung with an aneurism needle and tied. The lung was then cut off distal to the ligature. One-third of the animals died from cessation of respiration during the operation. Attempts to remove the right lung were only rarely successful because the right heart dilated when the pulmonary vessels were tied. The surviving rats recovered promptly from the anesthetic and in a very short time were apparently well.

Two groups of controls of the same sex and age were used. In the first no operation was performed, while in the second all the steps of the operation with the exception of the ligature and removal of the lung were completed. The nitrogen content of the right lung of these control rats was compared with the nitrogen content of the remaining right lung of the experimental group.

An interval of from 60 to 61 days was allowed to elapse from the time of operation and then both the experimental and the control animals were anesthetised, several mg. of heparin dissolved in Locke's solution were injected by a needle and syringe into the circulation through the heart, the chest cavity was widely opened, the left auricle incised, a glass cannula thrust into the right ventricle, and Locke's solution run slowly into the still beating heart. As a rule the pink

flow of blood through the remaining lung. The conflicting histological observations have been summarized by Kawamura (6). He himself observed an increase in the elastic tissue and capillaries in the alveoli of the remaining lung.

lung tissue blanched white at once but in some cases, which were rejected, appreciable quantities of blood were left. When the perfusion fluid ran clear from the left auricle and no more blood could be seen in the lung, the right lung was excised in a uniform manner, cutting the bronchus and vessels as closely as possible to the lung. After blotting on filter paper the entire lung was transferred to a Kjeldahl flask and digested for 3 hours with 30 cc. H_2SO_4 , 10 gm. K_2SO_4 , and 0.7 gm. Hg.

The left lung of the albino rat was found by Jackson to weigh approximately 0.33 times the total lung weight (7). The results

TABLE I.
Comparison of the Nitrogen of the Right and Left Lungs.

Right lung	Left lung	Both lungs	Ratio: $\frac{\text{Left lung } N_2}{\text{Both lungs } N_2}$
mg	mg	mg.	
19 12	11 94	31 06	0 384
17 56	9 79	27 35	0 358
15 54	7 42	22 96	0 323
15 47	8 49	23 96	0 354
15 28	8.16	23 44	0 348
15 28	8 48	23 76	0 357
14 66	8 07	22.73	0 355
14 62	8 27	22 89	0 361
13 72	7 56	21 28	0 355
Average... ..			0.355

given in Table I in which the nitrogen content of the right and left lungs are compared show that the left lung contains on the average 0.36 times the total lung nitrogen.

In the experimental rats, therefore, whose left lung was excised, only about one-third of the total lung tissue was removed, whereas when a kidney, adrenal, testicle, or ovary is excised the total organ tissue is approximately halved. This difference invalidates any comparison of the degree of lung hypertrophy with the degree of hypertrophy in other paired organs. However it will probably be found possible to get about half the lung tissue out by removing first one lobe of the right lung and later the whole of the left lung.

The left lung was removed from twenty-seven rats, but operative deaths, failures to wash blood completely from the lung, and the pneumonia of rats which has been described by Hektoen (8), reduced the number of successful experiments to twelve. The same causes brought the operated control group down to twelve. Pneumonia was found in 24 per cent of the animals. All cases were rejected in which it was present in no matter how slight a degree in either lung.²

TABLE II.

Comparison of the Nitrogen Content of the Right Lung in the Control Rats without Lung Excision and in the Experimental Rats Whose Left Lung Had Been Excised.

First controls: no operation	Second controls: left lung exposed	Experimental: left lung excised	Difference between second control and experimental rats
Right lung N ₂	Right lung N ₂	Right lung N ₂	Control = 100 per cent
mg.	mg.	mg.	per cent
19.12	19.40	28.32	+46.0
17.56	17.02	27.74	+63.0
15.54	16.74	26.74	+59.8
15.47	16.47	24.92	+51.5
15.28	16.22	23.11	+42.5
15.28	15.96	22.97	+43.9
14.66	15.62	22.82	+46.2
14.62	15.36	20.72	+35.0
13.72	14.14	20.14	+42.5
	14.02	19.38	+38.2
	13.94	18.33	+31.5
	13.67	18.33	+34.1
Average. 15.7	15.7	22.8	+45

The results are given in Table II, in which the absolute amounts of N₂ found are recorded and in Table III, where the N₂ is expressed

² In one control animal it happened that the whole of the left lung was consolidated and reduced to about one-fourth of its usual size, while the right lung was free from pneumonia. A nitrogen determination showed that the right lung contained 33.78 mg. N₂, more than twice the average nitrogen content of the right lung of the controls. In several other instances of unilateral disease a high N₂ content was found in the other lung so that it would seem that an increase in the mass of the remaining healthy lung tissue occurs as a result of lung disease in the rat.

as mg. per sq. dm. of body surface (body surface = 11.4 times the two-thirds power of the body weight).

In both control groups the amount of N_2 in the right lung averaged 15.7 mg., while the average N_2 of the one lung rats was 22.8 mg. Relatively to body surface the controls had 3.5 and 3.8 mg. N_2 per sq. dm., while the experimental rats had 5.5 mg. N_2 per sq. dm. of

TABLE III.

Comparison of the Nitrogen Content of the Right Lung Per Sq. Dc. of Body Surface in the Control Rats without Lung Excision and in the Experimental Rats Whose Left Lung Had Been Excised.

First controls: no operation	Second controls: left lung exposed	Experimental: left lung excised	Difference between second control and experimental rats
Right lung N_2	Right lung N_2	Right lung N_2	Control = 100 per cent
mg. per sq.dm.	mg. per sq.dm.	mg. per sq.dm.	per cent
4.64	4.91	6.72	+36.9
3.96	4.05	6.31	+55.8
3.75	4.05	6.28	+55.1
3.65	4.00	6.27	+56.8
3.38	3.86	6.15	+59.4
3.23	3.81	5.82	+52.8
3.15	3.78	5.18	+37.0
3.08	3.64	5.04	+38.5
2.98	3.61	4.78	+32.5
	3.58	4.58	+28.0
	3.43	4.55	+32.6
	3.41	4.36	+27.9
Average. 3.5	3.8	5.5	+43

body surface. When the operated control and experimental results are arranged in order of magnitude and compared individually there is in every instance a marked increase in the N_2 of the one lung rats. The average increase is 45 per cent when the N_2 is given in absolute terms and 43 per cent when it is expressed relatively to body surface.

CONCLUSION.

In the albino rat the removal of the left lung, which constitutes about one-third of the total lung tissue, is followed by an average

increase of more than 40 per cent in the mass of the remaining lung, as estimated from the nitrogen content of the almost blood-free tissue.

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THE PRECIPITIN REACTION OF ANTIPNEUMOCOCCUS SERA.*

I. THE PRECIPITIN INDEX.

BY HARRY SOBOTKA, PH.D., AND MAE FRIEDLANDER.

(From the Department of Bacteriology, New York University and Bellevue Medical College, New York.)

(Received for publication, September 2, 1927.)

The study of the precipitating reaction between antipneumococcus sera and the soluble specific substance derived from the three fixed types of *Diplococcus pneumoniae* promises a quantitative method for measuring the antibodies present in antipneumococcus sera. The relative simplicity in the chemical composition of one of the constituents, namely the soluble specific carbohydrate, paves the way for making this immunological phenomenon better understood.

There is little doubt that the mutual compensation of electrical charges between two reacting colloids causes the phenomenon of precipitation (1, 2). So far colloidal chemistry offers an explanation for the mechanism of the reaction but not for the cause of its specificity toward that part of the colloidal serum constituents commonly known as antibodies. Avery, Heidelberger, and Goebel (3) demonstrated that the type specificity of the antigen is accounted for by distinct differences in chemical composition between the soluble specific substance of the three fixed types. The carbohydrates with their variability of configuration are apt to cause great physiological specificity, e.g. in the enzymatic hydrolysis of saccharides.

On the other hand, the knowledge of the chemical nature or even of the concentration of the active specific principle of the other participant, the antibody, is so obscure that a differentiation of types from this angle seems futile at the present time.

We endeavored to establish mathematical laws for the precipitin

* This investigation was carried out by means of the Lucius N. Littauer Fund for Pneumonia Research.

reaction in the various types of pneumonia and thus from the dynamics of the specific reaction itself to obtain information concerning specificity.

When one adds various dilutions of antigen (precipitinogen)¹ to a constant dilution of antibody (precipitin) precipitation occurs until a certain dilution of antigen is reached. In the same way, when varying dilutions of antibody (precipitin) are added to a constant dilution of antigen (precipitinogen) precipitation occurs until a certain dilution of antibody is reached. The results of these reactions may be tabulated in charts as in Table I.

TABLE I.

Precipitin Reaction of Antipneumococcus Serum 32, Type I.

Dilution of serum	Dilution of soluble specific substance in millions											
	0.8	1.6	3.2	6.4	12.8	19.2	25.6	38.4	51.2	76.8	102.4	153.6
10	+	+	+	+	+	+	+	+	+	+	+	+
20	+	+	+	+	+	+	+	+	+	+	+	-
30	+	+	+	+	+	+	+	+	+	+	-	-
40	+	+	+	+	+	+	+	+	+	-	-	-
60	+	+	+	+	+	+	+	-	-	-	-	-
80	+	+	+	+	+	+	±	-	-	-	-	-
120	+	+	+	+	+	-	-	-	-	-	-	-
160	+	+	+	+	+	-	-	-	-	-	-	-
200	+	+	+	+	-	-	-	-	-	-	-	-
240	+	+	+	+	-	-	-	-	-	-	-	-
320	+	+	+	±	-	-	-	-	-	-	-	-
400	+	+	+	-	-	-	-	-	-	-	-	-
480	-	-	-	-	-	-	-	-	-	-	-	-

The sensitivity of this precipitation, as determined by the last plus sign (positive precipitation) in any horizontal or vertical row, may be computed by multiplication of the concentration of the soluble specific substance by the concentration of the antibody. Where the antibody concentration is increased the antigen may be proportionally decreased and still give a positive reaction; an eightfold dilution of an antibody solution, for example, requires an eight times greater concentration of the antigen to give precipitation compared with the antigen

¹ The terms "antigen" and "precipitinogen" are used in the following in the sense of a chemical reagent; no implication is made as to immunizing action (4).

concentration necessary to precipitate the original antibody solution. The mathematical expression for this inverse proportionality is the constancy of the above product which can be verified for the wide range of serum dilutions from 1:5 to 1:500. The product is constant within this range just like the so called "solubility product" in the case of inorganic ions forming insoluble compounds. An "ionic product" can be calculated for a mixture of solutions containing barium ions and sulfate ions by multiplying the concentration of the two. When this surpasses a certain value, commonly termed the solubility product, more barium sulfate is formed than can be kept in a dissolved state and precipitation occurs. The mathematical law for the appearance of the insoluble precipitate as observed in this example may be compared, to a certain extent, to that applying to the appearance of insoluble precipitate when antiserum is added to antigen. The mechanism of this combination is different from the barium sulfate formation in so far as the composition of the precipitated compounds is not constant as to the amounts of the two constituents. Colloidal reactions are not stoichiometrical and precipitates vary as to their composition as indicated by their different gross appearance. When the serum is in excess a voluminous precipitate is formed consisting of many very fine particles which stay in suspension for some time after shaking. On the other hand an excess of soluble specific carbohydrate will cause a precipitation of larger individual flakes which settle to the bottom more readily and stick together.

By testing the supernatants it may be shown that an excess of soluble specific substance removes the antibody beyond the point where it can be detected, but an excess of antibody fails to remove the soluble substance to this extent (see Table VIII, page 69) (12,13,14).

Absolute values for the solubility products of the specific precipitates cannot be given because the absolute value for one constituent only can be inserted. The soluble specific substance has a constant chemical composition which in pure state exhibits constant immunological activity. It may be used as a reproducible standard in evaluating the relative concentration of the antibody which is measured by the sensitivity of the precipitation with the former at maximum dilutions. The absolute concentration and activity of the antibody, however, are still unknown because it has not as yet been demonstrated free from other components of the serum.

It seems practical to substitute the exceedingly small values of the solubility product by their reciprocals. These are computed by multiplying the dilutions instead of the concentrations. As these figures were higher than a million their one-millionth part was used as the *Precipitin Index*. If this index were constant throughout each test it would be sufficient to look for it in one dilution of one constituent and to vary only the dilution of the other one. Different factors, however, bring about irregularities in the precipitin reaction.

As precipitation according to the accepted assumption is due to the mutual compensation of the electrical charges of a positive and a negative colloid, a large excess of either one is able to keep the colloid of opposite charge in solution. On this account a considerable amount of either antigen or antibody will fail to precipitate in the presence of an excessive concentration of the other. A phenomenon of a similar type, usually termed pro-zone, is met with in the agglutination of *B. typhosus* (5) and of *Pneumococcus*; a high concentration of serum fails to cause agglutination with a concentration of antigen which gives a positive reaction with lower serum concentrations. The opposite phenomenon was described for precipitation in Type II pneumonia by Morgan (6), *viz.* a rather high concentration of soluble specific substance did not react with antiserum in its lower concentrations although the latter did react with smaller amounts of the soluble specific substance. We designate this reversed action the post-zone. Zones are more frequently observed in Type III than in Type I.

Consideration of the ratio between soluble specific substance and serum reveals that the pro-zone occurs in the test-tubes where this proportion ranges between 80,000 and 320,000 for Type III and between 300,000 and 1,600,000 for Type I. A similar figure can be derived for the post-zone ranging between 1,250 and 6,250 for Type III and between 400 and 2,400 for Type I. Although both the pro-zone and post-zone do not appear in all sera, we are able, however, to observe both zones in the same serum. In such cases the diagram suggests a certain zone of positive precipitation between the limits given in the above figures.

The quotients $\text{sss dilution} / \text{serum dilution}$ or $\text{serum concentration} / \text{sss concentration}$ for the post-zone in Types I and III are about one-tenth of the corresponding values for the point at which the antibody

is entirely removed by precipitation (*cf.* Table VIII). A ten times greater amount of antigen than is necessary to precipitate one unit of antibody is required to keep it in solution.

The absolute concentration of antibody in serum is unknown. Tentatively let us assume that the concentration of the precipitin anti-

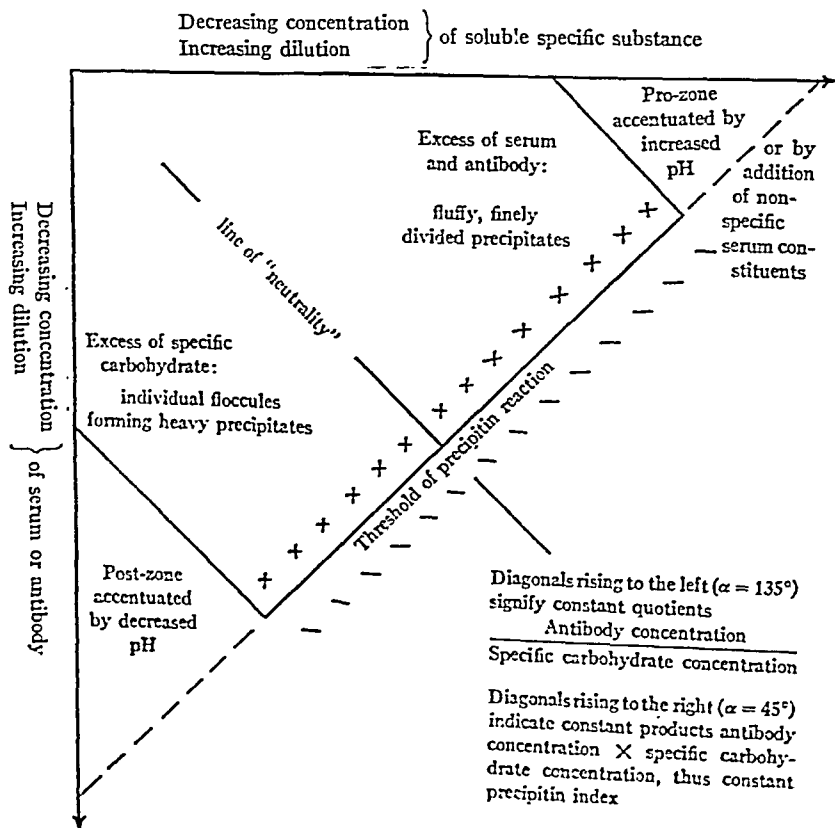


FIG. 1.

body in the serum is 1 in 20,000;² then the quotients antibody/sss attain 1/20,000 of the values antiserum/sss given above as character-

² According to a private communication from Dr. F. M. Huntoon a value between 0.054 and 0.018 per cent may be assumed for the percentage of antibody in the usual antipneumococcus sera.

istic of the two limiting zones. Hence for Type I the compound is insoluble between the limits of 15 to 80 parts antibody to 1 part sss on the one side, and of 1 part antibody to 8 to 50 parts sss on the other. For Type III the compound is insoluble in a fourfold to sixteenfold excess of either antibody or specific carbohydrate.

The range of insolubility in Type III seems from the above figures to be limited to a much narrower variation in the proportion of precipitin to precipitinogen than in Type I. In Morgan's experiment

TABLE II.
Ratio $\frac{\text{Concentration of Antiserum}}{\text{Concentration of SSS}}$ for Pro- and Post-Zones.

Zone and type	Range of variation	Extreme values		Number of observations	Average quotient
		Lowest	Highest		
Pro I	320,000-1,600,000	200,000 215,000	3,200,000	13	900,000
Post I	400-2,350	400	5,000	11	1,500
Pro III	80,000-320,000	50,000 60,000	2,560,000 1,280,000 960,000 640,000 (twice) 480,000	26 (18)	350,000 (210,000)
Post III	1,250-6,250	1,000	40,000 20,000 10,000	21 (17)	6,000 (3,000)

with Type II the figures indicate that this variation lies between I and III. These differences are partially if not entirely due to the differences in acid equivalents of the soluble specific substances. The quotient antibody/sss for the post-zone in Type III is three times that in Type I. In other words it requires one-third the excess of soluble specific substance III than I to prevent the homologous precipitation. According to their acid equivalents—340 for Type III and at least 600 for Type I—these solutions are about equimolecular.³ Similar

³ A report of experiments on the equivalent weight of soluble specific substance, Type I, will be given in another place.

considerations cannot yet be applied to the antibody because of the presence of a non-specific factor in the serum. Whether this factor whose manifestations will be discussed in the following paragraphs is an independent fraction of the serum globulin or associated with the antibody proper is a problem for future investigation.

Addition of Non-Specific Serum Fraction (cf. 7).—The antigenic soluble substance constitutes only a small fraction of the precipitate which by itself is only a fraction of the total solids present in the reacting fluids. By far the greater part of the precipitate originates in the serum. It is difficult to imagine that this overwhelming portion should be endowed with as high a degree of specificity per unit of weight as is the specific carbohydrate, unless a single specific group of the antibody is associated with a large protein molecule; in other words the antibody equivalent is very high. The following facts suggest that the association between the antibody proper and its protein carrier can be broken without irreversible inactivation of the antibody group: refined fractions obtained from antisera by the ammonium sulfate methods and containing the full amount of protective antibody, as proven by injection into the blood stream, at times do not show the precipitin reaction. This ability could be restored in some of our experiments by addition of normal serum.

A similar though less striking effect was observed with other purified antibody solutions and several original antisera, where, by the addition of normal serum as well as of the globulin fractions of normal serum or heterologous antisera, an inconspicuous precipitin reaction was increased as to its amount and as to its extent in the series of dilutions. Another general observation points to the complex nature of the antibody: the disappearance of the precipitin reaction in serum concentrations of 1 to 500 and below compared with the high sensitivity for low antigen concentration may be explained by the multi-molecular character of the reaction on the part of the serum.

In Serum 32, Type I, the addition of normal horse serum increased the sensitivity when the concentration of antibody was low, but it reduced the sensitivity causing a pro-zone when the concentration of the antiserum was high (Table III).

A general increase of the precipitin indices upon addition of normal serum to Serum 68, Type I, is recorded in Table IV.

TABLE III.

*Precipitation of Antipneumococcus Serum from Horse 32, Type I.
A. With Addition of 10 Per cent Normal Horse Serum.*

Dilution of antiserum	Dilutions of soluble specific substance in millions											
	0.04	0.2	0.4	0.8	1.6	3.2	6.4	12.8	25.6	38.4	51.2	102.4
20							+	+	+	±	—	—
40							+	+	+	±	—	—
60							+	+	+	+	—	—
80							+	+	+	±	—	—
100						+	+	+	+	—	—	
120						+	+	+	±	—	—	
160					+	+	+	+	—	—		
200					+	+	+	+	—	—		
240					+	+	+	±	—	—		
280					+	+	+	±	—	—		
320				+	+	+	+	—	—			
400				+	+	+	+	±	—			
480			+	+	+	+	+	—				
600			+	+	+	+	±					
800		+	+	—	—	+	+					
1200		+	—	—	+	+						
2400	—	—	—	—								

B. Without Addition of Normal Serum.

20							+	+	+	+	+	+
40							+	+	+	+	±	—
60							+	+	+	+	—	—
80							+	+	+	—	—	—
100						+	+	+	±	—	—	
120						+	+	+	±	—	—	
160					+	+	+	+	—	—	—	
200					+	+	+	+	—	—	—	
240					+	+	+	+	—	—	—	
280					+	+	+	—	—	—		
320					—	+	+	—	—	—		
400			—	—	—	—	—	—	—			
480			—	—	—	—	—	—	—			
600			—	—	—	—	—	—				
800			—	—	—	—	—	—				
1200		—	—	—	—	—						
2400		—	—	—	—	—						

An experiment tending to confirm the significance of this factor is the dominance of the pro-zone when two sera, only one of which shows a pro-zone, were used in a mixture of equal amounts. The result as given in Table V was not an average as one might expect, but practically the same as in the serum with the pro-zone. The significance of this frequently occurring condition upon the testing of pooled serum and similar mixtures is obvious.

The influence of buffers on the sensitivity and on the zonal phenomena of the precipitin test was investigated (*cf.* 6, 8). Of course, serum itself is a buffer. The pH of a serum buffer mixture will as a rule be found between the pH of the buffer and the pH of the serum. The value will approach the more concentrated and better buffer of the two.

TABLE IV.

Precipitin Indices for Serum 68, Type I, with and without Addition of 10 Per Cent Normal Horse Serum.

Dilution of antiserum	Precipitin index	
	With 10 per cent normal serum	Without 10 per cent normal serum
10	60	<20
20	80	<40
40	320	80
80	320	160
160	480	640
320	960	480

In a special experiment the influence of the specific precipitation on the pH of the serum was determined. As expected from the minute amount of precipitate (see page 63), it was found either constant or showed slight and irregular changes only.⁴

The following conclusions were drawn. Phosphates outside the natural range of reaction impair the sensitivity; citrate in the range used in our experiments does not act in this way. The same type of buffer at different pH causes the pro-zone to disappear and the post-zone to appear when going to the acid side. The development of a post-zone renders the reaction less sensitive. Although the actual

⁴ E. F. Hirsch (9) observed slight changes to the alkaline side in a rabbit anti-sheep precipitin system.

changes in pH are slight, they increased the power of the soluble specific substance to keep the antibody in solution, thus causing a post-zone. This can be attributed to the fact that the dissociation of the acid soluble carbohydrate is increased toward low pH making it possible to reach more readily an excess of the specifically active anions. When the reaction approaches the alkaline side, formation of electropositive

TABLE V.

Precipitin Test of Two Type III Sera, 125 and 126 and of Their Mixture.

No. and dilution of serum	Dilution of soluble specific substance in millions										P.r.
	0.4	0.8	1.2	1.6	3.2	4.8	6.4	9.6	12.8	25.6	
No. 125											
5	+	+	+	+	+	-	-	-	-	-	(16)
10	+	+	+	+	+	-	-	-	-	-	(32)
20	+	+	+	+	+	+	+	±	-	-	160
40	+	+	+	+	+	±	-	-	-	-	160
60	+	-	-	-	-	-	-	-	-	-	(24)
No. 126											
5	+	+	+	+	+	+	+	+	+	-	(64)
10	+	+	+	+	+	+	+	+	-	-	(96)
20	+	+	+	+	+	+	+	+	-	-	192
40	+	+	+	+	+	+	±	-	-	-	224
60	+	+	+	+	+	±	-	-	-	-	240
No. 125+126											
5	+	+	+	+	+	-	-	-	-	-	(16)
10	+	+	+	+	+	-	-	-	-	-	(32)
20	+	+	+	+	+	+	±	-	-	-	112
40	+	+	+	+	+	+	±	-	-	-	224
60	+	-	-	-	-	-	-	-	-	-	(24)

For parenthesized figures see Table VI of following paper.

ions from the antibody which probably has an ampholytic nature (10) is increased thus accounting for the more prompt appearance of a pro-zone by overcompensation of the negative charges of the anions of the soluble specific substance.

Practical applications of these facts are treated in the succeeding paper.

EXPERIMENTAL PART.

The antigenic material employed in our tests consisted of the type specific soluble substances prepared from broth cultures of pneumococci and purified by

TABLE VI.

Precipitin Index of Antipneumococcus Serum 32, Type I, from Test Recorded in Table I.

Dilution of serum	Greatest dilution of sss giving a+ (or =) precipitation	Precipitin index by multiplication of corresponding serum and sss dilutions
	<i>millions</i>	
10	102.4	1024
20	102.4	2048
30	76.8	2304
40	51.2	2048
60	25.6	1536
80	(25.6)	1792
120	12.8	1536
160	12.8	2048
200	6.4	1280
240	6.4	1536
320	(6.4)	1536
400	3.2	1280
480	<0.4	<192
		1722
Dilution of sss	Greatest dilution of serum giving a+ (or =) precipitation	
<i>millions</i>		
0.8	400	320
1.6	400	640
3.2	400	1280
6.4	(320)	1792
12.8	160	2048
19.2	80	1536
25.6	(80)	1792
38.4	40	1536
51.2	40	2048
76.8	30	2304
102.4	20	2048
153.6	<10	<1536
		1820

repeated precipitations; their ash was less than 0.5 per cent, the nitrogen of the Type I substance was 4.5 per cent by Kjeldahl.

Dilutions were made ranging from 1:250,000 to 1:150,000,000 in the case of

Type I and from 1:100,000 to 1:50,000,000 with Type III. The increase in the magnitude of these solutions was in geometric progression with an increment of 2, or $3/2$ and $4/3$ alternately.

The sera used were thoroughly centrifugalized at 2,000 to 2,400 revolutions per minute for 1 hour and the supernatant filtered before making up the dilutions. It was necessary to take these precautions because in many of the sera a sediment occurs which might easily be mistaken for a positive "thread" reaction. Normal serum, too, before centrifugalization gave what seemed to be positive results. Controls consisted of various dilutions of serum in physiological salt solution. Wherever these were not negative the test was discarded.

TABLE VII.

Determination of Precipitin Index of Serum 90, Type I.

Dilution of serum	Dilutions of sss Type I in millions											Precipitin index
	1	2	4	8	16	24	32	48	64	96	128	
5	+	+	+A	+	+	+	+	+	+	+	+	640
10	+	+	+	+	+	+	+	+	-	-	-	480
20	+B	+	+	+	+	+	+	+	-	-	-	960
40	+	+	+	+	+	-	-	-	-	-	-	640
60	+	+	+	+	+	-	-	-	-	-	-	960
80	+	+	+	+	-	-	-	-	-	-	-	640
160	+	+	+	-	-	-	-	-	-	-	-	640
240	+	+	±	-	-	-	-	-	-	-	-	720
320	+	+	-	-	-	-	-	-	-	-	-	640
480	-	-	-	-	-	-	-	-	-	-	-	(<480)
Average.....												725

For A and B see Table XII.

Series of twelve tubes ($2\frac{1}{2}$ by $\frac{3}{8}$ inches) were arranged in racks and marked with the figures of the increasing dilutions of the soluble specific substance of which 0.5 cc. was used. To each was added 0.5 cc. of the dilution of the serum. Thus each rack contained tubes with varying concentrations of soluble specific substance and a constant concentration of serum. These, together with controls, were placed in a water bath with 37°C . for 2 hours and then put into the ice box until the following morning when the reactions were read and recorded.

If a flaky precipitate was not visible at first the tubes were spun for a thread reaction. The use of ++, +++, etc. was discarded because it is impossible to indicate from the appearance of the precipitate the quantity actually present (6, 11). Doubtful tubes were recorded by the sign ±.

The method of calculating is illustrated in Table VI. The last dilution of the soluble specific substance giving a + reaction was multiplied by the serum dilution

used in this particular test (rack). If the last reaction is doubtful—such \pm reactions are recorded in the tables by parenthesized figures—the average value between it and the decidedly positive reaction to its left was inserted. Similar figures were obtained when the limit of sensitivity was taken from the vertical rows of equal sss concentration instead of the horizontal. An average was taken over the range of high sensitivity thus eliminating zonal effects.

In Table I and in Table VI antiserum from Horse 32, Type I, was used. The precipitin index — P.I. — from the horizontal rows was

TABLE VIII.

Precipitin and Precipitinogen in Supernatants of Precipitates from Polyvalent Antipneumococcus Serum 3335 with Soluble Specific Carbohydrates of Types I and III.

Precipitate formed from dilution of		To corresponding supernatant added equal amount of												
		Dilution of sss (millions)										Dilution of serum		
Serum	sss I			0.125	0.25	0.5	1	2	4	8	16	10	20	40
2.5	4,000,000			+	+	+	+	+	+	\pm	—	\pm	—	—
5	2,000,000			+	+	+	+	+	\pm	—	—	\pm	\pm	—
10	1,000,000			+	+	+	+	\pm	—	—	—	+	\pm	\pm
20	500,000			+	+	+	\pm	—	—	—	—	+	+	—
40	250,000			—	—	—	—	—	—	—	—	+	+	+
	sss III	0.025	0.05	0.1	0.2	0.4	0.8	1.6	3.2	6.4	12.8			
2.5	800,000	+	+	+	+	+	+	+	+	+	\pm	\pm	\pm	—
5	400,000	+	+	+	+	+	+	+	+	+	—	\pm	\pm	\pm
10	200,000	—	—	—	—	—	—	—	—	—	—	+	+	\pm
20	100,000	—	—	—	—	—	—	—	—	—	—	+	+	+
40	50,000	—	—	—	—	—	—	—	—	—	—	+	+	+

1,720, from the vertical rows 1,820. As a rule the P.I. was computed from the horizontal rows.

Table VII is another very regular test in which Antiserum 90, Type I, was used with a resulting P.I. of 725.

Serum 3335, a polyvalent serum obtained from a horse immunized against all three types of pneumonia gave regular results and was used to obtain approximate information as to the equivalence of the two reagents.

10 cc. of serum in concentrations of 2 $\frac{1}{2}$, 5, 10, 20, and 40 per cent were

mixed with 10 cc. of soluble specific substance Type I in dilutions of 4, 2, 1, $\frac{1}{2}$, $\frac{1}{4}$ million respectively. Another series with the same serum dilutions were made with soluble specific carbohydrate Type III in dilutions of 800,000, 400,000, 200,000, 100,000, and 50,000. The precipitates were centrifugalized after the usual incubation and the supernatants tested for remaining antibody or antigen. In the first four supernatants of the experiments with Type I where the serum

TABLE IX.

Examples for Pro-Zone and Post-Zone of Precipitation with Antisera from Type III Horses.

No. and dilution of serum	Dilution of soluble specific substance									Quotient Serum sss
	Millions									
	0.2	0.4	0.8	1.2	1.6	3.2	4.8	6.4	9.6	For pro-zone
(a) No. 302										
20	+	+	+	+	+	—	—	—	—	80,000
40	+	+	+	+	+	+	—	—	—	80,000
80	+	+	+	+	+	+	+	+	—	80,000
160	+	+	+	+	+	+	±	—	—	—
320	—	—	—	—	—	—	—	—	—	—
	Thousands									For post-zone
	24	32	40	48	64	96	128	256	512	
(b) No. 127										
20	+	+	+	+	+	+	+	+	+	—
40	+	+	+	+	+	+	+	+	+	—
60	—	—	±	±	+	+	+	+	+	667
80	—	—	—	—	—	+	+	+	+	800
120	—	—	—	—	—	—	+	+	—	800
160	—	—	—	—	—	—	—	—	—	—

concentration was 25,000 or more times that of the soluble specific substance and in the first two supernatants of the Type III series where this excess was 80,000 fold, antibody was present. The subsequent combinations with a serum/sss quotient of 6,250 for Type I and 20,000 for Type III did not contain detectible amounts of antibody.⁵

An analogous experiment was performed, testing the supernatant

⁵ Morgan (6) gives a ratio 1:10,000 to 1:20,000 soluble specific substance: antiserum for maximum precipitation in the Type II system.

with the original serum. Only those supernatants found free from antibody gave a considerable reaction with soluble specific substance; the others revealed the presence of slight amounts of non-precipitated antigenic substance by a faint reaction (12, 13, 14).

The ten original precipitates were resuspended in physiological salt solution and combined with undiluted serum or 0.1 per cent soluble specific substance. Some of the precipitates changed their appearance but none redissolved.

Table IX (a) gives a pro-zone for pooled Serum 302, Type III, the zone being characterized by a serum/sss quotient of 80,000. An ex-

TABLE X.

Precipitin Test of Antibody Preparation 102, Type I, with Soluble Specific Substance in Dilutions 0.4, 0.8, 1.6, 3.2, 6.4, 12.8, 25.6, 38.4, 51.2, 102.4 Million, in Presence of 10 Per Cent Normal Horse Serum.

Dilution of preparation	Greatest dilution of sss giving + (or =) reaction	Precipitin index	Quotient $\frac{\text{Serum}}{\text{sss}}$
	<i>millions</i>		<i>pro-zone</i>
80	(51.2)	3584	560,000
120	25.6	3072	215,000
160	51.2	8192	320,000
200	51.2	10,240	
240	(51.2)	9216	
320	25.6	8192	
400	(12.8)	3840	
480	12.8	6144	
600	6.4	3840	
800	(12.8)	7680	
1200	(1.6)	1440	

periment for the post-zone phenomenon is given in Table IX (b) carried out with Serum 127, Type III, against the homologous specific substance. The simultaneous occurrence of a post-zone (serum/sss = 3,750) and a pro-zone (serum/sss = 960,000) are shown in Table XIV (a) (page 75).

Preparation 102 from pooled antisera Type I precipitated in the presence of 10 per cent normal horse serum as shown in Table X. Without this addition it did not exhibit any precipitin power at all.

The precipitation as a whole was not very strong but reached high dilution. There is no doubt as to the existence of a pro-zone. Table III, for which the numerical values of the p.i. are given in Table XI presents the influence of normal serum on Antiserum 32; its sensitivity is increased and a pro-zone with a serum/sss of 600,000 and 800,000

TABLE XI.

Precipitin Test of Antiserum 32, Type I, Same Sample as Recorded in Table III.

(A) *With Addition of 10 Per Cent Normal Horse Serum.*

(B) *Without This Addition.*

Dilution of antiserum	Greatest dilution of sss giving +		Precipitin index		Quotient $\frac{\text{Serum}}{\text{sss}}$	
	(A)	(B)	(A)	(B)	(A)	(B)
20	(38.4)	102.4	640	2048	1,600,000	
40	(38.4)	(51.2)	1280	1792	800,000	
60	38.4	38.4	2304	2304	640,000	
80	(38.4)	25.6	2560	2048		
100	25.6	(25.6)	2560	1920		
120	(25.6)	(25.6)	2304	2304		
160	12.8	12.8	2048	2048		
200	12.8	12.8	2560	2560		
240	(12.8)	12.8	2304	3072		
280	(12.8)	6.4	2688	1792		
320	6.4	6.4 <u>1.6</u>	2048	2048		5000
400	(12.8)	<0.4	3840	<160		
480	6.4		3072			
600	(6.4)		2880			
800	6.4 <u>1.6</u>		5120		2,000	
1200	3.2 <u>0.8</u>		3840		667	
2400	<0.04		<96			

The underscored bold faced figures give greatest dilution of sss giving -- reaction in post-zone.

is produced. Another example is given in Table IV, page 65, showing the influence of 10 per cent normal horse serum on Type I Antiserum 68.

The influence of buffers on the actual pH of Type I Antiserum 90 was measured potentiometrically and it was found that the deviations from the pH of the buffer itself are smaller in the range where the buffer has the highest buffer value (15). For the same buffer mixture a

smaller deviation was caused by the addition of 5 per cent serum than of 40 per cent serum.

Precipitation of these mixtures with soluble specific substance using

TABLE XII.

pH of Mixtures of Equal Amounts of Buffers and Serum 90, Type I, and Influence of Precipitation upon pH of Buffered Serum for Combinations Marked A and B in Table VII.

Buffer		pH by potentiometric determination				
		Buffer alone	Serum dilution 2.5	A Serum dilution 5 sss dilution 4 million	Serum dilution 10	B Serum dilution 20 sss dilution 1 million
Phosphates (M/15)						
Primary	Secondary					
10.0	0.0	4.56	6.23	6.19	5.68	—
9.5	0.5	5.53	6.37	6.37	—	—
9.0	1.0	5.83	6.27	—	6.02	5.92
7.0	3.0	6.38	6.71	6.57	6.43	6.39
4.0	6.0	6.91	7.08	7.17	6.89	6.90
1.5	8.5	7.46	7.55	7.61	7.42	7.24
0.5	9.5	7.88	7.88	7.60	7.60	7.33
Citrate NaOH (N/10)						
10.0	0.0	4.92	5.20	5.30	4.98	4.97
8.0	2.0	5.35	5.72	5.77	5.37	5.42
6.0	4.0	5.98	6.72	7.05	6.24	6.24
Borate (N/10)		9.10	—	—	9.00	—
Serum 90		Undiluted 7.72	—	—	{ 7.66 7.58	—

Precipitates in phosphate mixtures more voluminous, with citrate "10.0" very slight.

a dilution of 4 million for serum dilution 2.5 and 1 million for serum dilution 20 caused slight variations as given in Table XII.

If a certain phosphate mixture is added to varying concentrations of serum the pH for low serum concentration will approach the original

TABLE XIII.

Influence of Phosphate Buffers upon Precipitin Test of Antipneumococcus Serum 3534, Type III. (a)

Greatest dilutions of soluble specific substance III giving + (or \pm) precipitation, in millions. Dilutions used: 0.3, 0.4, 0.6, 0.8, 1.6, 3.2, 6.4, 12.8, 25.6, 51.2.

A. Final concentration of phosphate N/20 throughout the test							
Dilution of serum	pH of buffer	4.6	5.5	6.1	6.8	7.3	8.0
	pH of mixture with serum dilution 10	—	—	—	7.13	7.38	7.45
10		(25.6)	12.8	25.6	(6.4)	3.2	3.2
20		6.4	(25.6)	(6.4)	6.4	3.2	3.2
40		3.2	6.4	3.2	(3.2)	<0.8	0.8
80		(3.2)	1.6	0.8	(3.2)	<0.2	<0.2
160		<0.2	<0.2	<0.2	<0.2	—	—
80 post-zone		0.8	0.2	0.4	(3.2)	—	—
40	Greatest dilution of sss giving — (or \mp) precipitation	—	—	—	(0.8)	—	—
Serum sss for post-zone		10,000	2,500	5,000	20–40,000	—	—

B. Original serum diluted with N/4 phosphate

Dilution of serum	Concentration of buffer	pH of buffer	(b) 4.6	5.5	6.1	6.8	(c) 7.3
		pH of mixture with serum dilution 10	6.37	6.50	6.63	7.05	7.53
5	N/20		25.6	12.8	51.2	12.8	(12.8)
10	N/40		(25.6)	12.8	51.2	12.8	12.8
20	N/80		6.4	6.4	(12.8)	6.4	6.4
40	N/160		6.4	6.4	6.4	(6.4)	6.4
80	N/320		1.6	(3.2)	(3.2)	(3.2)	3.2
160	N/640		(1.6)	1.6	(1.6)	1.6	3.2
160 post-zone	As above		0.2	—	0.4	(0.2)	—
Serum sss for post-zone			1,250	—	2,500	1,000	1,920,000 for pro-zone

(a) For test of Serum 3534 without buffer see Table XIV (a).

(b), (c) Compare Table XIV (b, c).

pH value of the phosphate. If this pH is extreme no reaction takes place. This occurred with phosphate buffers of a pH less than 5.0 and more than 6.5 for serum concentrations of 1:40 and below. Even in higher serum concentrations alkaline phosphates will impair the sensitivity, as seen in Table XIII, A, which gives the effect of phosphates upon precipitation of Serum 3534, Type III.

TABLE XIV.

Influence of Hydrogen Ion Concentration on Precipitin Reaction of Type III Serum 3534.

Dilution of serum	Dilution of soluble specific substance in millions									Precipitin index
	0.2	0.4	0.8	1.6	3.2	6.4	12.8	25.6	51.2	
(a) Without buffer										
5				+	+	±	—	—	—	24
10				+	+	+	±	—	—	96
20			+	+	+	+	—	—		128
40			+	+	+	±	—	—		192
80	—	±	+	+	—	—				128
160	—	—	—	—	—	—				—
(b) Phosphate 4.6										
5				+	+	+	+	+	—	128
10				+	+	+	+	±	—	192
20			+	+	+	+	—	—		128
40			+	+	+	+	—	—		256
80	+	+	+	+	—	—				128
160	—	+	+	±	—	—				192
(c) Phosphate 7.3										
5				+	+	+	±	—	—	48
10				+	+	+	+	—	—	128
20			+	+	+	+	—	—		128
40			+	+	+	+	—	—		256
80	+	+	+	+	+	—				256
160	+	+	+	+	+	—				512

The pH can, however, be maintained constant in a test by diluting the buffer parallel to the serum. Thus, in Table XIII, B, where the original serum is diluted *after* addition of $\frac{1}{4}$ N phosphates even the slight variations in the resulting acidity show pronounced effects on the diagram. The sensitivity of the reaction was increased toward the

alkaline side and at the same time a post-zone characteristic of the serum disappeared. On the acid side a higher sensitivity in the greater serum concentrations suppressed the pro-zone.

When using citrate of pH 5.0 a post-zone appeared and the sensitivity increased for a Type III serum as compared with a control without any buffer or with serum phosphate mixture more alkaline than the citrate.

TABLE XV.

Influence of Buffers on the Precipitin Reaction between Antiserum 125, Type III, and Homologous Soluble Specific Substance.

Dilutions of sss: 50,000; 100,000; 200,000; 400,000; 800,000; 1,600,000; 2,400,000; 3,200,000; 4,800,000. Figures in table are "greatest dilutions" in thousands.

Dilution of serum	Buffers used with final concentration and pH					Without buffer	
	Citrate N/20		Phosphate M/30				Borate N/20
	5.0		6.0	7.0	8.0		9.0
5	1600		1600	1600			
10	1600		1600	1600	1600	1200	1600
20	1200	Post-zone	2400	(4800)	2400	1200	3200
40	800	<u>200</u>	800	(1600)	1600	800	(800)
60	(800)	<u>200</u>	<200	800	<50	(200)	800
100	1200	<u>400</u>	—	<200	—	—	<50
160	<200		—	—	—	—	—
Quotient Serum SSS	Post-zone 5000 3333 4000		Pro-zone 320,000 160,000			—	160,000

The underscored bold faced figures indicate zonal phenomena.

The action of citrate on Serum 170, Type I, is shown in Table XVI. When using this buffer a pro-zone was observed on the alkaline side. The post-zone was moved towards lower serum and higher soluble specific substance concentrations, the quotient serum/sss shifting from 2,350 to 400 for pH 6.0; in other words the ability of the soluble specific substance to keep the compound unprecipitated decreases towards alkaline reaction.

SUMMARY.

The mechanism of the precipitin reaction between antipneumococcus sera and the type-specific soluble carbohydrate is investigated. The sensitivity of the reaction is found to be generally constant when expressed by the product of the concentrations of the two reacting substances. Precipitin index (P.I.) is defined as one-millionth of the reciprocal value of this product.

TABLE XVI.

Influence of Citrate Buffers on Precipitation of Serum 170, Type I.

For precipitation without buffer see Table VII.

sss I dilutions: 1/16, 1/8, 1/4, 1/2, 1, 2, 4, 8, 16, 24, 32, 64 million.

Dilution of serum	Greatest dilution of sss showing + (or =) reaction (For post-zone: - (or ≠) reaction)					
	pH	4.7	5.0	5.3	5.7	6.0
5		64	(64)	64	16	16
10		64	(32)	(32)	16	(24)
20		24	16	24	16	12
40		16	(16)	(12)	24	12
80		8 <u>(1/4)</u>	8	(8)	(8)	8
160		8 <u>1/4</u>	(8) <u>(1/4)</u>	4 <u>1/4</u>	4	(4)
240		<1/16	<1/16	<1/16	1 <u>(1/8)</u>	2 <u>1/8</u>
320		—	—	—	1 <u>(1/4)</u>	(1) <u>1/8</u>
Quotient Serum sss	Pro-zone	—	—	—	3,200,000 1,600,000 800,000	3,200,000
	Post-zone	2350 1550	1150	1550	400 600	500 400

For explanation of underscored bold faced figures see Table XI.

Zonal phenomena and their bearing on the absolute concentration and the equivalent weight of the antibody are discussed. The greater tendency towards exhibition of a post-zone in Type III is connected with the lower acid equivalent of the homologous specific carbohydrate.

The influence of the addition of normal serum and the influence of slight changes in pH are studied. The addition of normal serum as

well as the increase in pH promote the pro-zone, whereas decrease in pH promotes the post-zone. The sensitivity of the reaction is accordingly influenced by the pH in different ways depending on the range of concentration.

The precipitin index allows the recognition and elimination of zonal irregularities. Thus it offers a method for the standardization of pneumonia antibody.

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THE PRECIPITIN REACTION OF ANTIPNEUMOCOCCUS SERA.*

II. THE RATIO OF PRECIPITIN TO PROTECTIVE ANTIBODY

BY MAE FRIEDLANDER,† HARRY SOBOTKA, PH.D., AND EDWIN J. BANZHAF, PH.D.

(From the Department of Bacteriology, New York University and Bellevue Medical College, and the Research Laboratory, Department of Health, New York.)

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In the diagnosis of infectious disease precipitin and agglutinin reactions have assisted in the identification of the invading organism, when the patient's serum has been tested with suspected organisms or when antigenic material from the patient (1) has been tested against antisera.

Agglutination has been used also in the quantitative determination of the antibody in antityphoid and antimeningococcus sera. For the standardization of diphtheria antitoxin Ramon (2; see also reference 3) suggested a test-tube method based on the formation of a flocculent precipitate when toxin and antitoxin were combined in definite proportions.

The present paper records investigations concerning the parallelism of the precipitin with the protective antibody in antipneumococcus sera. The quantitative determination of antibody in antipneumococcus sera is based on the relative reactivity of the precipitin and the protective antibody; the question of their identity is not raised.

Agglutination tests in pneumonia require the use of uniform cultures; they thus share with the animal protection test the disadvantage of possible biological irregularities. Precipitin tests, however, are carried out with a reproducible non-cellular antigen of a definite chemical composition.

* This investigation was carried out by means of the Lucius N. Littauer Fund for Pneumonia Research.

† This communication is part of a thesis to be submitted by Mae Friedlander in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Bacteriology at New York University.

Method.

The precipitin indices for a number of antipneumococcus sera and antibody preparations were determined by the precipitin tests described in the preceding paper. The study of the zonal phenomena permits the elimination of these irregularities.

Only the higher values furnished by multiplication of serum and precipitinogen dilution were used in calculating the precipitin indices, all individual values smaller than one-half of the maximal value were disregarded in estimating the average of each test. Precipitin index is a millionth part of the product obtained by mul-

TABLE I.

Comparison of Precipitin and Protective Antibodies in Monovalent Type I Sera and Antibody Preparations.

No. of serum	Precipitin index	Protective units	Ratio	Reference
155	400	100	4.0	Table IX (d)
47	560	200	2.8	Table IX (d)
169	1250	200-400	4.1	Table IV
90	700	200	3.5	Table VII preceding paper
32	1720	400	4.3	Tables I and VI preceding paper
32	2550	800	3.2	Tables III and XI preceding paper
	2880†		3.6	
94	3840	800	4.8	Table IV
Preparation "I"	2340	800	2.9	Table IV
102	7170†	800	9.0	Table X preceding paper
916	1920	200	9.6	See page 88
Average ratio for the first nine values.....			3.8	

† With addition of 10 per cent normal horse serum.

tiplying the dilution of the antiserum by the dilution of the antigen. For details see the preceding paper.

The proportions of the corrected precipitin index to the protective units of the same antibody solution were compared and an unanticipated constant relation was revealed.

The quotient precipitin index/protective units for a number of monovalent Type I horses ranged between 2.8 and 4.8 with an average of 3.7. For some monovalent Type III horses the

TABLE II.

Precipitin and Protective Antibodies in Monovalent Type III Sera.

No. of serum	Precipitin index	Protective units	Ratio	Reference
125	160	4, 8, 10	16-48	Table V preceding paper
	192			Table V
126	220	4	34-55	Table V preceding paper
	135			Table V
127	128	4	32	Table V
128	85	4-10	8-21	Table V
302	575	10	58	Table X
"A"	770	10	77	Table X
"B"	6150	80	77	Table X
Average ratio of all values except the two last			34	

TABLE III.

Relation between Precipitin Index and Protective Potency for Polyvalent Antisera.

Horse No	Duration of treatment	Type I			Type III		
		P. I.	P. U.	Ratio	P. I.	P. U.	Ratio
	<i>mos</i>						
3079	28	720	1000	0 72	>340		
3180	28	530	500	1 06	320	10	32
			1000	0 53		(20)	(16)
3332	22	530	400	1 35	>340	10	34
			500	1 06		(20)	(17)
3335	22	350	<500	>0 70	>190	(8)	(24)
3514	11	400	100	2 0	72	2	36
			200	4 0		(4)	(18)
3530	10	480	200	2 4	256	10	26
						(20)	(13)
3531	10	60	50	1 2	>192		
3635	6	240	50	2 4	45	2	23
			100	4 8			
3640	6	60	50	1 2	30		
3639	6	<40	50	<0 8	50	2	25
Average ratio { upper 4 horses				0 9			24
{ lower 6 horses				2 3			

The parenthesized figures for protective units in Type III are based on 72 hours delay of death rather than on survival over 96 hours; on account of the pro-zone familiar in Type III pneumococcus mouse tests no survivals occurred.

quotient ranged between the extremes of 8 and 58 with an average of 34 (Tables I and II). When comparing these figures with those of Type I the almost ten times greater precipitin power associated with the protective potency in Type III is striking. If we discount this difference by referring the higher precipitin index per protective unit in Type III to the higher specific activity of equal weights of the precipitinogen rather than of the antibody, there is seen to be a similar ratio between precipitin and protective activity of the antisera for the two types investigated.

For a number of polyvalent horses¹ decidedly lower values for the quotient precipitin index/protective units were observed. In Type I they ranged between 0.7 and 2.4 with an average of 1.75 and with extreme values of 0.53 and 4.8. For Type III in polyvalent horses the quotient ranged among the lower values of those given above for monovalent sera of this type, the average being 24 (Table III).

A decrease in precipitin activity was also observed when adding one heterologous monovalent serum to an equal amount of another.

Serum 90, Type I shows a precipitin index of 700 and upon the addition of Serum 129, Type II, the precipitin index was only 60. Serum 126, Type III, decreased upon addition of No. 129 from 135 to 32.

If the Type I values of the polyvalent horses were arranged according to the duration of the treatment they could be divided into two groups; one comprising four horses under pneumococcus treatment for 2 years had quotients ranging below 1.35 with an average of 0.9, while another group of horses under treatment for no longer than 1 year had values above 1.2 with an average of 2.3.

This observation indicates that the duration of the treatment influences the proportion of the two antibody activities. This became evident from an experiment on two Type I horses whose immunization we observed from the onset. It was found that the quotients for bleedings after 37 days were about twice as high as those after 114 days; the protective potency reached greater values at a slower rate than the precipitin.

When the precipitin test was applied to refined antibody prepara-

¹ Samples of these were supplied through the kindness of Dr. Stanley Beard of the Lederle Antitoxin Laboratories, Pearl River, New York.

tions the quotients obtained were higher than from the crude sera in our experiments. We observed values as high as 9 in Type I and above 70 in Type III. There is some doubt whether this difference arises during the refining process by a selective fractionation or is simply due to a lesser proportionalism between potency and concentration in concentrated solutions.

When making due allowance for the various influences noted and

TABLE IV.

Precipitin Test of Antipneumococcus Sera, Type I, No. 94 and No. 169 and of Antibody Preparation "f" Prepared by L. D. Felton's Procedure (4).

Serum No.	Dilution of serum	Dilution of soluble specific substance in millions									Precipitin index	
		4	8	12	16	24	32	64	96	128	"P. I."	
94	20				—	—	—	—	—	—	(<320)	
					(pro-zone)							
	40				—	—	—	—	—	—	(<640)	
	80				+	+	+	±	—	—	3840	
					(post-zone)						3840	
	120	—	—	—	—	—	+	—			3840	
169	20				+	+	+	+	—	—	1280	
	40				+	—	—	—	—	—	640	
	80				—	—	—	—	—	—	<1280	
	120	+	+	+	—	—	—	—			1440	
	160	+	+	+	—	—	—	—			1920	
	240	—	—	—	—	—	—				(< 960)	
"f"	20				+		+	±	±	+	2560	
	40				+		+	+	—	—	2560	
	60				+		±	±	—	—	1920	
	120				—		—	—	—	—	(<1920)	

discussed in the present and in the preceding paper a fairly accurate idea of the amount of antibody contained in an antipneumococcus serum can be obtained. The determination of the precipitin index may replace the slow mouse test in the course of immunizing horses as well as during refining processes; for final controls animal tests will have to be retained.

EXPERIMENTAL PART.

In the following tables data are collected for several monovalent Type I and Type III sera. Some of them like No. 94, Type I, Table IV, and No. 125 and No. 127, Type III, Table V, show a pro-zone and

TABLE V.
Precipitin Test of Antisera 125 to 128, Type III.

Dilution of serum	Dilution of soluble specific substance III in millions															P. I.
	0.05	0.1	0.2	0.3	0.4	0.6	0.8	1.2	1.6	2.4	3.2	4.8	6.4	9.6	12.8	
No. 125																
10	+	+	+	+	+	+	+	+	+	-	-	-				(16)
20	+	+	+	+	+	+	+	+	+	±	-	-				(40)
40	-	+	+	+	+	+	+	+	+	+	+	+				192
80		±	-	+	-	-	-									(24)
120		-	-	-	-	-	-									-
No. 126																
10	+	+	+	+	+	+	+	+	+	+	+	+	+	+	±	112
20	+	+	+	+	+	+	+	+	+	+	+	+	+	±	-	160
40	±	+	+	+	+	+	+	+	±	±	-	-				(64)
80		-	-	-	+	±	-									(40)
120		+	-	-	±	-	-									(42)
No. 127																
10	+	+	+	+	+	+	+	+	+	-	-	-				(16)
20	+	+	+	+	+	+	+	+	+	-	-	-				(32)
40	+	+	+	+	+	+	+	+	+	+	+	-				128
80		+	+	+	-	+	-									(48)
120		-	-	-	-	-	-									-
No. 128																
10	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	64
20	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	96
40	±	-	+	+	+	+	+	+	+	+	-	-	-	-	-	96
80		+	±	-	-	±	-									(40)
120		±	+	+	±	+	-									72

in these instances only the high values obtained from higher serum dilutions were taken into consideration. Values lower than 50 per cent of the highest value in a test are parenthesized and were disregarded.

In Table VI experimental results for Type I and Type III of ten

polyvalent horses, immunized against three types, are given. The figures are the dilution products or precipitin indices for the individual serum dilutions given in the first column. Pro-zones of more or less extent, may be observed in Sera 3079, 3180, 3640 and others for both types, in Sera 3514 and 3530 for Type I, and almost in all sera for serum dilution 1:5 for Type III.

TABLE VI.

Synopsis of Precipitin Tests of Sera Obtained from Ten Polyalent Horses (Types I, II, III).

Precipitin indices for single serum dilutions given. Figures in parenthesis disregarded in computation of average P.I.

Dilution of serum	Horse No.									
	3079	3180†	3332	3335†	3514	3530	3531	3635	3640	3639
Type I										
5	(80)	(40)	—	320	(30)	(20)	40	(120)	(<20)	Less
10	(80)	(80)	—	320	(120)	(60)	60	(80)	(<20)	than
20	(80)	(160)	(240)	320	(160)	(160)	40	(40)	60	40
40	(160)	320	320	320	(160)	(160)	80	(80)	60	
80	480	640	640	320	320	320	(<40)	240	(<40)	
160	960	640	640	480	480	640	(<80)	240	(<80)	
Type III										
									‡	‡
5	(64)	(>128)	—	>128	—	>128	>128	32	(8)	32
10	(64)	(96)	—	192	—	>256	>256	32	32	48
20	(48)	(128)	—	>256	64	256	192	64	32	64
40	256	256	256	256	64	384	(64)	(<24)	28	28
80	>256	192	>256	128	64	256	(128)	(<16)	(<16)	16
160	>512	>512	>512	(96)	96	(96)	(<32)	—	—	<32)

† see Table VII. ‡ see Table VIII.

Examples for both regular and pro-zone sera of each type are given in Tables VII and VIII.

A synopsis of these values is given in Table III, page 81, where the precipitin indices as calculated from Table VI are listed in the third and sixth columns. The protective units in the fourth and seventh columns of Table III, and in the third columns of Tables I and II were obtained from mouse tests, carried out according to the government standards in the Research Laboratories, Department of Health, City of New

TABLE VII.

Reaction of Soluble Specific Substance Type I with Polyvalent Antipneumococcus Sera.

Serum 3335 (Very Regular Test).

Dilution of serum	Dilution of soluble specific substance in millions								
	0.5	1	2	4	8	16	32	64	128
5				+	+	+	+	+	—
10				+	+	+	+	—	—
20			+	+	+	+	—	—	
40			+	+	+	—	—	—	
80	+	+	+	+	—	—			
120	+	+	+	±	—	—			

Serum 3180 (Pro-Zone Type).

5				+	+	—	—	—	—
10				+	+	—	—	—	—
20			+	+	+	—	—	—	
40			+	+	+	—	—	—	
80		+	+	+	+	—	—		
120		+	+	+	—	—	—		

TABLE VIII.

Reaction of Soluble Specific Substance Type III with Polyvalent Antipneumococcus Sera.

Serum 3639 (Regular).

Dilution of serum	Dilution of soluble specific substance in millions							
	0.2	0.4	0.6	0.8	1.6	3.2	6.4	12.8
5				+	+	+	+	—
10				+	+	+	±	—
20			+	+	+	+	—	
40			+	±	—	—	—	
80	+	—	—	—	—			
120	—	—	—	—	—			

Serum 3640 (Pro-Zone).

5				+	+	—	—	—
10				+	+	+	—	—
20			+	+	+	—	—	
40			+	±	—	—	—	
80	—	—	—	—	—			
120	—	—	—	—	—			

TABLE IX.

Development of Precipitin and Protective Antibodies against Type I Diplococcus Pneumoniae in Two Horses.

Dilution of serum	Precipitin indices for individual serum dilutions. Duration of immunizing treatment.							
	(a) 0		(b) 37		(c) 72		(d) 114 days	
	47	155	47	155	47	155	47	155
10			(60)	(120)	(<40)	(<40)	(30)	(160)
20			(80)	160	(80)	(40)	(40)	(60)
40	Nil Nil		(120)	240	(160)	(80)	(240)	(80)
80			640	320	480	320	640	320
160			640	(<160)	480	640	480	480
Average P.I.			640	240	480	480	560	400
Protective units	Nil Nil		80-100	40	80	100	200	100
Ratio	—		6.4-8.0	6.0	6.0	4.8	2.8	4.0

TABLE X.

Precipitin Test of Serum 302, Type III, and of Two Globulin Fractions A and B, Concentrated 16 Times and 14 Times Compared with the Original Serum 302.

Dilution of anti-body solution	Dilution of precipitinogen in million-												P. I.
	0.2	0.4	0.8	1.2	1.6	3.2	4.8	6.4	9.6	12.8	25.6	51.2	
No. 302													
20	+	+	+	+	+	—	—	—	—	—	—	—	(32)
40	+	+	+	+	+	+	—	—	—	—	—	—	(128)
80	+	+	+	+	+	+	+	+	—	—	—	—	512
160	+	+	+	+	+	+	±	—	—	—	—	—	640
320	—	—	—	—	—	—	—	—	—	—	—	—	—
A													
80	+	+	+	+	+	+	+	—	—	—	—	—	(384)
160	+	+	+	+	+	+	—	—	—	—	—	—	(512)
320	+	+	+	+	+	+	—	—	—	—	—	—	1024
480	—	—	—	—	+	—	—	—	—	—	—	—	768
B													
160	+	+	+	+	+	+	+	+	+	+	+	±	6144
320	—	—	—	+	+	+	+	—	—	—	—	—	(1536)
480	+	+	—	—	+	+	—	—	—	—	—	—	(1536)
A+B													
160	+	+	+	+	+	+	+	+	+	+	+	±	Stronger reactions than B alone

York, by Miss G. M. Cooper under the direction of Dr. W. H. Park, and in the Testing Department of the Lederle Antitoxin Laboratories, Pearl River, New York, by Miss F. L. Clapp, to whom we have to express our thanks for their kind cooperation.

The influence of the duration of the treatment on the quotient precipitin index/protective power was studied in two monovalent horses, No. 47 and No. 155, for which the experimental data are to be found in Table IX. The quotients were 6 to 8 after 5 weeks, 4.8 to 6.0 after 10 weeks and 2.8 to 4.0 after 16 weeks.²

Two globulin fractions were obtained by the ammonium sulfate method (6) from Serum 302 (Table X). The first was concentrated to one-sixteenth, the second to one-fourteenth of the original volume. The first fraction contained only ten mouse units per cc. like the starting material, in the second a concentration of 80 units was attained. The ratio precipitin/protection, already as high as 58 in the original serum, rose to 77 for the globulins. By mixing the two globulin fractions in equal amounts an effect was observed similar to those discussed on page 65 of the preceding paper.

The precipitin values for Preparation 916, Type I, 200 protective units (Table I), were

for a serum dilution of 10	1280
20	2560
40	160
80	80
160	160;

in this preparation, as in No. 102 of Table VI in the preceding paper, the precipitin activity was very high as compared with the protective potency.

SUMMARY.

The precipitin indices for a number of monovalent and polyvalent antipneumococcus sera were determined under known conditions, and found to vary as did the number of protective units.

The ratio precipitin index/protective units in monovalent sera was

² Forster (5), investigating the relation between the time elapsed since immunization and the precipitin titer, observed the development of a post-zone during the "secondary stage."

found to lie between 2.8 and 4.8 for Type I and to be about ten times greater for Type III.

Lower values were found in polyvalent horses and when mixing heterologous monovalent sera with each other.

The influence of the duration of treatment upon the quotient was studied.

Several refined and concentrated preparations showed a relative increase in precipitin activity.

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THE ANTIGENIC COMPLEX OF STREPTOCOCCUS HÆMOLYTICUS.

I. DEMONSTRATION OF A TYPE-SPECIFIC SUBSTANCE IN EXTRACTS OF STREPTOCOCCUS HÆMOLYTICUS.

By REBECCA C. LANCEFIELD, PH.D.

(From the Hospital of The Rockefeller Institute for Medical Research.)

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Chemical and immunological studies to determine the nature of the substances responsible for the specific reactions of a number of microorganisms have been reported recently by several investigators. Most of this work has been reviewed in some detail elsewhere (1). In certain very carefully studied instances, such as pneumococcus (2) and the Friedländer bacillus (3), specificity is determined by type-specific carbohydrates, while non-specific group reactions are due to species-specific proteins. The same general relationship holds for the *Streptococcus viridans* (4).

Hitchcock (5) found that nearly all hemolytic streptococci yielded in crude antiformin extracts a "residue antigen," such as described by Zinsser and Parker (6), which reacted with all of his antibacterial sera prepared against hemolytic streptococci but not with sera against green streptococci. Conversely (7) similarly prepared "residue antigens" from non-hemolytic streptococci did not react with his hemolytic streptococcus antisera.

Hirsch (8) also reported finding a precipitating substance in extracts of hemolytic streptococci which he called the soluble specific substance. He did not give any evidence to support this statement other than the fact that it precipitated antibacterial sera and sera prepared against solutions of this material. He did not state whether his sera and antigens were all prepared and tested with one or with several strains.

In repeating the work of Hitchcock as a preliminary to the present experiments, it was found, indeed, that all partially purified antiformin extracts, or extracts prepared in such a way as to remove the bulk of the protein, precipitated equally well antibacterial sera made against

different types of hemolytic streptococci. The precipitates formed typical discs like those obtained with type-specific carbohydrates in other species of bacteria. All attempts to separate a type-specific fraction from such extracts by fractional alcoholic precipitations were unsuccessful. Methyl and ethyl alcoholic extracts of whole bacteria contained the same species-specific substance but no trace of a type-specific fraction.

It was then found that extracts made by Porges' method (9) for the removal of bacterial capsules contained a type-specific fraction. This report is the presentation of some of the work carried out with this fraction.

The strains used were chiefly those which had been previously grouped by agglutination and protection tests (10). These had been preserved for 4 or 5 years by dessicating them while in the frozen state by Swift's (11) technique. Cultures recovered from this stock, as a rule, retained the virulence and cultural and serological behavior characteristic of them at the time of dessication. These old strains were used in preference to freshly isolated ones because the distinctive types had already been separated and grouped. The results obtained by any new method of grouping must, of necessity, agree with those obtained by previous methods in order to establish its validity.

Methods.

Antigens were prepared from the various strains by a modification of Porges' method as follows: bacteria centrifuged from 18 hour plain broth cultures, were suspended in 0.85 per cent NaCl solution to which sufficient N/1 HCl was added to make a final concentration of N/20 HCl. Usually the sediment from 1 liter of broth culture was extracted in 10 cc. to 15 cc. volume. The suspension in a Pyrex centrifuge tube was immersed in boiling water for 15 minutes with occasional stirring, then cooled, centrifuged, and the supernatant fluid removed and neutralized with N/1 NaOH. The precipitate which appeared on neutralization was thrown down in the centrifuge; and the water-clear, slightly yellowish supernatant fluid was used as antigen for precipitin tests. When a more potent antigen was desired, it was obtained by extracting in smaller volume or by concentrating the dilute extract described above. For concentration, crystals of sodium acetate—usually 10 gm. per liter of extract—were added to facilitate the subsequent precipitation with three or four volumes of 95 per cent alcohol. After standing overnight in the ice box, the alcoholic precipitate was thrown down and redissolved in salt solution. The greater part of the alcoholic precipitate was insoluble; but

precipitin tests showed that very little loss of active material was occasioned by discarding the insoluble part. The alcoholic precipitation was repeated and the

TABLE I.
Precipitin Reactions with Unabsorbed Antibacterial Serum from Rabbit Q867, Immunized with Strain S60, Type S60.

Antigen HCl extract			Read after 2 hrs at 37°C	Read after additional 18 hrs in ice box
Strain	Type	Cc		
S60	S60	0 4	+++	+++
		0 3	+++	+++
		0 1	++	++
S43	"	0 4	++	+++
		0 3	+++	+++
		0 1	++	++
S23	S23	0 4	—	=
		0 3	—	+
		0 1	±	++
S65	"	0 4	—	+
		0 3	—	+±
		0 1	±	++
S3	S3	0 4	—	±
		0 3	—	+
		0 1	±	++
S144	"	0 4	—	±
		0 3	—	+
		0 1	±	++
S24	Unclassified	0 4	—	±
		0 3	—	+±
		0 1	±	++
S276	"	0 4	—	=
		0 3	—	+
		0 1	±	++

In all tables ±, +, ++, +++, +++++, indicate degrees of reaction; — indicates a negative reaction; 0 indicates that the test was not made.

antigen concentrated to any desired volume by this means. In the following experiments, most of the antigens were the original extracts, although a few were concentrated by the alcoholic precipitation method.

Antibacterial sera were prepared by inoculating rabbits intravenously with increasing doses of heat-killed broth cultures followed by living organisms. After 6 or 8 weeks of immunization, the sera of such animals contained agglutinins and precipitins and, in some instances, protective antibodies for the homologous strain. Precipitins for antigens from strains of unlike type were also present in most of these sera. Experiment 1 gives the typical precipitin reactions of the kind of antibacterial serum which gave only moderate cross-reactions with heterologous antigens.

TABLE II.

Precipitin Reactions with Unabsorbed Antibacterial Serum from Rabbit Q609, Immunized with Strain S23, Type S23.

Antigen: HCl extract			Read after 2 hrs. at 37°C.	Read after additional 18 hrs. in ice box
Strain	Type	Cc.		
S23	S23	0.4	++++	++++
		0.1	+++	+++
S65	"	0.4	+++	+++
		0.1	++	++
S60	S60	0.4	+	++
		0.1	+±	++
S128	"	0.4	+±	++
		0.1	++	++±
S3	S3	0.4	—	±
		0.1	+	++
S144	"	0.4	—	±
		0.1	+	++
S24	Unclassified	0.4	++±	++±
		0.1	+++	+++
S276	"	0.4	±	++
		0.1	+	+±

Experiment 1.—Eight HCl extracts were made from two strains of each of three types of hemolytic streptococcus and from two unclassified strains. Three dilutions of each antigen were set up as follows: 0.4 cc., 0.3 cc., and 0.1 cc. were placed in successive tubes, and the volumes made up to 0.4 cc. with salt solution. 0.1 cc. of serum from Rabbit Q867, immunized as indicated in Table I, was added to each tube; and, after mixing, the tubes were incubated in a 37°C. water bath for 2 hours. The tests were read immediately, and again after standing overnight in the ice box. Controls of serum alone, of antigens alone, and of antigens with normal serum were negative.

Table I shows that antigens of the type homologous to the serum formed heavy flocculent precipitates almost as soon as they were mixed with the serum. The maximum reaction with these antigens was reached during the initial incubation at 37°C., and the intensity changed very little during the ensuing period in the icebox. Heterologous antigens, on the contrary, precipitated the serum slowly, often forming no visible precipitate during the 2 hour period in the water bath, but, after standing overnight in the ice box, they gave disc precipitates similar to those formed by specific carbohydrates of some species of bacteria. The optimum zone for disc precipitates was usually in considerably higher dilutions than for the more nearly type-specific flocculent precipitates.

Experiment 2.—A similar experiment was performed with a slightly different series of antigens and antibacterial serum from another rabbit, immunized as shown in Table II. The same series of tests was set up as in Experiment 1, except that the tube containing 0.3 cc. of antigen was omitted.

The reactions shown in Table II were typical of another kind of antibacterial serum with which immediate pronounced cross-reactions were obtained. Considerably less specificity was evident in the precipitin reactions in this instance than in Experiment 1, in which the 2 hour reading seemed quite type-specific; but, in Experiment 2, this reading showed a large amount of cross-reaction. On the basis of this test the classification of Strains S60 and S128 was doubtful; and Strain S24 would have been placed in *Type* S23 if other evidence had not been available. No conclusion could be drawn from the character of the precipitate in these instances, since it had much the same appearance for the heterologous antigens as for the homologous. Some other means of distinction was necessary.

Accordingly, isolation of the type-specific antigen was attempted but was only partly successful. Fractional alcoholic precipitations served to separate the non-type-specific disc-forming substance; but no satisfactory method was devised for removing the substance which gave non-type-specific flocculent precipitates with immune serum; hence attempts were made to prepare type-specific antisera by absorption. Table III shows such an experiment.

Experiment 3.—Serum from Rabbit Q309, immunized as shown in Table III, was absorbed with a heterologous hemolytic streptococcus. The bacteria from 1.5 liters of plain broth culture of a heterologous strain were centrifuged, resuspended in a small volume of salt solution, and killed by heating at 56°C. for 1 hour. To the packed bacteria, 2 cc. of immune serum diluted with 4 cc. of salt solution were added. A parallel absorption was performed at the same time with bacteria from the strain homologous to the serum. Controls of immune serum and of normal serum similarly diluted were included, and the absorption carried out at 37°C. for half an hour. After centrifugation the clear supernatant diluted serum was removed and preliminary precipitin tests made. Since precipitates were no longer obtained with heterologous antigens, the absorption was considered complete. Table III shows the precipitin tests with a number of homologous and

TABLE III.

Absorption Experiment: Precipitin Reactions with Serum from Rabbit Q309, Immunized with Strain S23, Type S23.

Antigen: HCl extract		Not absorbed	Absorbed with heterologous Strain S60
Strain	Type		
S23	S23	+++	+++±
S65	"	++±	++
S60	S60	+	—
S43	"	++	—
S128	"	++	—
S4	"	++	—
S24	Unclassified	++	—
S276	"	+	—
R28	"	++	—

heterologous HCl antigens, and the heterologous absorbed serum, also the control unabsorbed serum. 0.2 cc. of serum dilution (equivalent to 0.07 cc. of undiluted serum), and 0.2 cc. of antigen were mixed, and incubated in a 37°C. water bath for 2 hours. Readings were made after an additional 18 hours in the ice box. All necessary controls were negative.

Table III shows that the serum absorbed with a heterologous strain had become, in effect, a type-specific serum. This absorbed serum reacted only with antigens of the type used in immunization, while reactions with heterologous antigens were all completely negative. The heterologous strain had, therefore, not only absorbed the anti-

bodies for all strains of its own type but also the antibodies for other heterologous strains. The control lot of unabsorbed serum gave good reactions with most of these antigens. Absorption of the same serum with the homologous strain removed the antibodies for the homologous antigen also. In view of these results it was evident that Strain S24 did not belong to *Type S23*, as might have been supposed from

TABLE IV.

Absorption Experiment: Precipitin Reactions with Serum from Rabbit Q612, Immunized with Strain S23, Type S23.

Antigen: HCl extract		Not absorbed	Absorbed with hemolytic streptococcus of				
Strain	Type		Homologous type:	Heterologous type:			
			S65, Type S23	S128, Type S60	S144, Type S3	S24, unclassified	New York 5, scarlatinal
S23	S23	++±	—	++	++	++±	++±
S65	"	+++	—	+++	+++	++	++
S39	"	+++	—	++±	+++	+++	+++
S60	S60	++	—	—	—	—	—
S6	"	+±	—	—	—	—	—
S128	"	++	—	—	—	0	0
S4	"	++	—	—	—	—	±
S43	"	++	—	—	—	—	—
S72	"	++	—	—	—	0	0
S3	S3	+	—	—	—	—	—
S80	"	+	—	—	—	—	—
S144	"	+	—	—	—	—	—
S149	"	+	—	—	—	—	—
S24	Unclassified	++±	—	—	—	—	—
S276	"	±	—	—	—	0	0

the heavy precipitation with unabsorbed serum from Rabbit Q609 (Table II).

Numerous similar absorption experiments were performed with different sera and with different hemolytic streptococci as absorbing agents; essentially similar results were obtained in all these experiments. Titration of absorbed serum with varying dilutions of heterol-

ogous antigens showed that shifting of the prozone was not responsible for the negative results. Finally, a more comprehensive experiment was performed.

Experiment 4.—Five aliquot portions of serum from Rabbit Q612 were absorbed respectively with five different strains of *Streptococcus hæmolyticus*, and a sixth portion kept as an unabsorbed control. Only one strain was the type homologous

TABLE V.

Absorption Experiment: Precipitin Reactions with Serum from Rabbit Q867, Immunized with Strain S60, Type S60.

Antigen: HCl extract		Not absorbed	Absorbed with hemolytic streptococcus of				
Strain	Type		Hemologous type:	Heterologous type:			
			S128, Type S60	S65, Type S23	S144, Type S3	S24, unclassified	New York 5, scarlatinal
S23	S23	+	—	—	—	—	—
S65	"	+±	—	—	—	—	—
S39	"	+	—	—	—	—	—
S60	S60	+++±	—	++±	++±	++	+++
S6	"	++	±	++	++	++	+++±
S128	"	++	—	—	+	0	+
S4	"	++	—	+	+±	+±	++
S43	"	+++	—	++	++	++	+++±
S72	"	+++±	—	+	++	0	++
S3	S3	±	—	—	—	—	±
S80	"	+	—	—	—	—	±
S144	"	+±	—	—	—	—	—
S149	"	+	—	—	—	—	—
S24	Unclassified	+	—	—	—	—	±
S276	"	+±	—	—	—	0	—

to the serum; three others were known heterologous types; the fifth belonged to an unclassified group. Absorption was accomplished with one or two 1 hour incubations, and was proved to be complete by preliminary testing with HCl extracts from heterologous strains. After being completely absorbed these sera were tested with HCl extracts from a number of strains representing different types (see Table IV). Readings with the absorbed sera made after 2 hours incubation at 37°C. and 18 hours in the ice box agreed. Controls of serum and antigen alone, and of antigen with normal serum, were negative.

A similar absorption experiment was performed with serum from Rabbit Q867, immunized with another type (see Table V).

This experiment showed again that unabsorbed antibacterial sera reacted with most antigens made from heterologous strains, although they reacted more strongly with homologous antigens than with heterologous. Absorption with bacteria of the homologous strain, or with bacteria of any strain of the same type, removed all antibodies; while absorption with any heterologous strain removed antibodies for *all* types of heterologous hemolytic streptococci, but left the type-specific antibodies practically intact. By this method, therefore, it was possible to prepare a serum which contained only type-specific antibodies.

Certain technical difficulties were encountered in these absorption experiments: complete absorption was often hard to attain because the serum could not be much diluted if it were to be used subsequently for satisfactory precipitin tests. Non-type-specific antibodies were absorbed more readily by certain strains than by others. Strains of the homologous type were more efficient in this respect than heterologous strains; and heterologous strains varied somewhat among themselves. Of the heterologous strains used in these experiments, S24 was the most effective absorbing agent. Various other workers have observed that some strains of bacteria are better than others for absorbing antibodies; and Krumwiede, Cooper, and Prevost (12) point out, in their comprehensive paper on agglutinin absorption, that this is often true. The present experiments with hemolytic streptococci indicate also that few absorptions with heavy emulsions are preferable to often repeated ones with fewer organisms. This is due partly to unknown factors, but can be partially explained by the additional dilution occasioned by using wet bacteria and also by the prolonged heating involved in repeated absorptions. Numerous absorptions with heterologous strains also tend eventually to reduce somewhat the titer of the type-specific antibodies. This may be due to a non-specific adsorption, such as occurs with kaolin, rather than to a lack of immunological specificity in the absorption process.

Examples of such overabsorption were usually found in sera which contained large amounts of non-type-specific antibody and conse-

quently required excessive absorption for its removal. Even such repeatedly absorbed sera still reacted, though in less degree, with most homologous antigens and not at all with heterologous. Such instances emphasize the importance of selecting sera with as little non-type-specific antibody as possible as well as the necessity of proper selection of the absorbing strain.

From three strains it was impossible to obtain a type-specific fraction by any of the methods which were usually successful. In one instance this was associated with loss of agglutinability; although in two others the strains were still agglutinable. While these were all old laboratory strains, so also were most of the other strains used in these experiments. It is possible that certain strains never possessed the function of producing a type-specific antigen; but it seems more probable, especially in view of the earlier successful classification of these strains, that some such factors as length of time under cultivation caused this condition. A study of other known groups of hemolytic streptococci might furnish a solution of this question.

DISCUSSION.

A type-specific substance was detected in HCl extracts of hemolytic streptococci by the absorption method. Such extracts also contained non-type-specific substances which gave confusing cross-reactions in the precipitin test when unabsorbed immune serum was used. Homologous antigens usually formed heavy flocculent precipitates as soon as they were mixed with the serum; while heterologous antigens often formed no precipitate until after the tubes had been in the ice box overnight. These non-type-specific precipitates were discs like those characteristic of type-specific carbohydrates of other species. Occasionally, however, non-type-specific precipitates appeared early and had the same flocculent characteristics as those formed by homologous antigens. Such results precluded the possibility of obtaining reliable type-specific precipitin reactions unless either the non-type-specific substances could be removed from the antigen or the non-type-specific antibodies from the serum.

While complete purification of the antigen by fractional precipitation proved extremely difficult or impossible, the preparation of type-specific serum by absorption was found to be easy. Although absorp-

tion with any strain of the homologous type removed all antibodies from the serum, absorption with bacteria from heterologous strains of hemolytic streptococci removed only the non-type-specific antibodies with the result that the serum no longer gave cross-precipitations with extracts from any heterologous strain of hemolytic streptococcus but still reacted with extracts from homologous strains, usually with only slight change in intensity. Numerous absorptions with heterologous bacteria eventually reduced the titer of type-specific antibodies; but it is probable that this is a non-specific adsorption, in the category of adsorption of antibodies by substances like kaolin, rather than an invalidation of the other experiments reported. Even in these extreme instances, however, type-specific reactions were still obtained with the absorbed serum. It was possible, therefore, to prepare antibacterial sera which contained only type-specific antibodies and consequently gave only type-specific precipitin reactions.

Of nineteen strains of hemolytic streptococcus studied, ten yielded type-specific antigens, six were tested for non-type specific antigens only since no homologous serum was available in these instances, and three failed to produce type-specific antigens. It seems probable that this failure was associated with long cultivation in the laboratory, or with other unknown factors, since these strains had been classified several years before by other methods. The classification of all other strains by the method described here agreed with that previously determined for these strains by agglutination and protection tests.

Obviously this has distinct advantages for classification of hemolytic streptococci over that possessed by the agglutination reaction. So many strains of hemolytic streptococci agglutinate spontaneously that the only methods previously applicable to their grouping were agglutinin absorption or serum protection of animals with which they were inoculated. Both methods are costly in time and material. Moreover, protection tests with strains of low virulence are impossible or inconclusive. The complexity of the antigenic structure of certain hemolytic streptococci makes the interpretation of results of agglutinin absorption at times very difficult, as shown recently by several authors (13). The application of this method to such groups might reveal a type-specific element in these strains, whereas the agglutinin absorption method has failed to indicate sharply defined types but has led to the suggestion by some of these authors of an antigenic mosaic.

The precipitin test, in contrast with the agglutination and protection tests, is applicable to any strain. The HCl extract can be prepared directly from the sediment of broth cultures in a few hours; and even when requiring additional concentration this can be completed in a day or two. The absorption of the non-type-specific antibody from the serum is the only time-consuming part of the method. Once absorption is complete, however, the sera may be kept in the ice box for months and used as required. The facility with which certain strains absorb non-type-specific antibodies is noteworthy and makes desirable the conservation of these types for this special purpose.

SUMMARY.

1. Hydrochloric acid extracts of *Streptococcus hæmolyticus* contain type-specific, as well as non-type-specific, substances. The precipitates formed by these crude extracts with homologous antibacterial serum are flocculent, while those obtained with heterologous serum are usually disc-like.

2. The type-specific substance may be detected by the use of antibacterial sera absorbed with heterologous strains of hemolytic streptococci. Such absorbed sera are type-specific: they are precipitated only by extracts of strains of the homologous type.

3. Any heterologous strain of hemolytic streptococcus absorbs the antibodies for all other heterologous strains, but homologous strains absorb type-specific antibodies as well. Numerous repeated absorptions with heterologous hemolytic streptococci tend to lower the titer of the type-specific antibody. A possible explanation of this fact is suggested.

4. Three strains did not yield a type-specific substance; and it seems probable that they had lost this function because of long continued cultivation in artificial media.

5. Classification based on the precipitin test with absorbed serum agrees with that previously determined by agglutination and protection tests. The method is, therefore, applicable to the problem of classification of the hemolytic streptococci.

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CHANGES IN THE PERCENTAGE OF CALCIUM AND PHOSPHORUS OF THE BLOOD FOLLOWING SECTION OF THE SYMPATHETIC AND VAGUS NERVES.*

By BENJAMIN N. BERG, M.D., ALFRED F. HESS, M.D., AND ELIZABETH SHERMAN.

(From the Department of Pathology, College of Physicians and Surgeons, Columbia University, New York.)

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That calcium and potassium, as well as other ions, are capable of stimulating or depressing the activity of nerves has been known for many years, having been established by the brilliant studies of Jacques Loeb as well as others. Recently the conception of the interaction between nerves and inorganic salts of the body has been amplified so as to include a reciprocal relationship between the two—not only an effect of ions on the function of the nerves, but of nerve impulses on the distribution of various ions.

The latter conception has been emphasized especially by the recent work of Kraus and Zondek (2) who suggested that the nerves exert their specific functions by means of the various ions, that, for example, the sympathetic system has an action similar to calcium or rather to an artificial excess of calcium. Elaborating this idea somewhat further Zondek (3) stated that the ions are the very materials made use of by the nerves to fulfill their functions, that stimulation of the vagus leads to a preponderance of potassium, whereas similar stimulation of the sympathetic leads in turn to the preponderance of calcium. The nerves are supposed to control not only the distribution of these ions within the cells and cell membranes, but the hydrogen ion balance as well. At about the same time Billigheimer (4) brought forward a similar point of view and showed that stimulation of the sympathetic nerves by means of suparenin induced lowering of the calcium level of the blood, a result which was interpreted as due to a local increase of calcium within the area of stimulation. Pilocarpin is stated to have had the opposite effect in relation to calcium, but in this regard the interpretation seems questionable.

* Presented at the Annual Meeting of the American Society of Biological Chemists, Rochester, April 14, 1927 (1).

In association with studies of the calcium and phosphorus metabolism in rickets and tetany, it seemed of interest to determine whether the concentration of these ions would be appreciably affected by lesions of the sympathetic and parasympathetic systems. Accordingly, a series of experiments were carried out in which the great abdominal centers of the sympathetic system were largely destroyed or the vagi were severed within the abdomen. The percentage of calcium and inorganic phosphate of the blood was then closely followed for prolonged periods. In this connection it may be mentioned that in 1867 Eckhard (5) reported that by cutting the splanchnic nerves he was able to bring about the appearance of sugar in the urine, a manifestation which was, however, not constant and which he attributed to a stimulative effect. Recently Nakao (6) sectioned the splanchnic nerves in rabbits in order to study the effect on the excretion of urine; he concluded that this lesion decreased diuresis and lessened the percentage of chlorides in the blood.

Operative Procedure.

All of the operations were performed on dogs under complete ether narcosis. Exposure of the nerves was accomplished by means of an upper median abdominal incision, except in relation to the left splanchnic which in some instances was exposed by the lumbar route. At every operation, in addition to division of the nerve trunks, traction was made and a segment 2 to 3 cm. in length was resected in order to preclude the possibility of regeneration. The sympathetic ganglia and plexuses likewise were resected and were examined histologically for verification of the tissue. The operations were performed with a minimum degree of exposure and handling of the viscera.

Operations on the Splanchnic Nerves.—The right splanchnic was exposed solely by the intraperitoneal route. The left splanchnic was exposed either intraperitoneally or extraperitoneally. These nerves were isolated below the internal arcuate ligaments of the diaphragm. In order to identify them, it was found of value to follow cephalad the branches to the suprarenal glands and then to expose the main trunk by traction.

Operations on the Celiac and Superior Mesenteric Plexuses and Ganglia.—The method which was found most satisfactory for the removal of these structures was preliminary isolation of the hepatic and celiac arteries, and separation of the right celiac ganglion. The dissection was followed to the aorta and continued caudad along its ventral surface and to the left of the vena cava, extending to the superior mesenteric artery, so that the superior mesenteric and left celiac ganglia were also separated. All of the intervening tissue lying between the suprarenals

was excised, care being taken to avoid injury to the glands. Careful dissection of the retroperitoneal tissues was necessary in order to avoid the receptaculum chyli and the larger lacteals. As large an area as possible was included in the resection of the plexuses and ganglia, which were removed *en masse*. The amount removed varied from case to case.

Experiments on the Abdominal Sympathetics.

A considerable number of operations of this kind were carried out. The results are summarized in Table IV. Characteristic data of

TABLE I.

Calcium and Inorganic Phosphate of the Blood Following Partial Removal of the Celiac Plexus.

Dog 3	Date	Interval after operation	Blood		Remarks
			Inorganic P	Serum Ca	
	1926		mg.	mg.	
Mongrel ♀ 9 kg.	12/8		2.87	10.7	Previous to operation
		3 hrs.	3.79	9.1	Diet: bread and cooked meat
	12/9	25 "	3.87	7.5	
		25½ "	2.47	6.4	Injected intravenously 0.1 mg. epinephrine (in 1 cc. 0.85 per cent NaCl)
	12/10	49 "	3.26	6.8	
	12/11	68 "		6.9	
	12/13	5 days	2.76	5.9	Temperature 38.5°. Blood CO ₂ 58.8
	12/14				
	12/16	8 "	3.69	6.8	Blood sugar 85.1 mg.
	12/21	13 "	4.27	6.4	" " 92.7 "
	12/23	15 "	3.16	6.7	" " 93.4 "
	1927				
	1/4	27 "	4.09	11.2	Intermittent fever and nasal discharge
	1/5				Weight 9 kg. Killed. Diffuse bronchopneumonia

operations in which the celiac plexus was partially removed, or the right or left splanchnic nerves divided, are reproduced in Tables I, II, and III. A review of these tables shows that the calcium level of the serum fell consistently following destruction of the celiac plexus or of the splanchnic nerves. In the first case which is charted, the percentage reached the low level of 5.9 mg. It is of in-

terest to note that in spite of this low level of calcium, the animal evinced no signs of tetany. The Kramer-Tisdall method was used for

TABLE II.

Calcium and Inorganic Phosphate of the Blood Following Division of Right Splanchnic Nerve.

	Date	Interval after operation	Blood		Remarks
			Inorganic P	Serum Ca	
Dog 6	1927		mg.	mg.	
Mongrel	1/4		2.63	11.0	Previous to operation
♀		2 hrs.	5.58	8.2	Diet was bread and cooked meat
6 kg.	1/5	25 "	3.02	6.0	
	1/11	7 days	4.28	7.8	
	1/21	17 "		10.35	Galvanic reactions: cathodal closing 2.5-3.0, opening 10.5
Dog 7					
♂	1/11		2.03	10.0	Previous to operation*
		2½ hrs.	2.27	6.9	Diet was bread and cooked meat
	1/12	24 "	2.35	7.3	
	1/21	9 days		8.3	
	1/22				Galvanic reactions: cathodal closing 1.9, opening 9.5
	1/31	19 "	2.10	10.2	

* 2 cm. of the nerve was resected.

TABLE III.

Calcium and Inorganic Phosphate of the Blood Following Division of Left Splanchnic Nerve.

Dog 9	Date	Interval after operation	Blood		Remarks
			Inorganic P	Serum Ca	
	1927		mg.	mg.	
♂	1/24		2.51	9.8	Previous to operation
8 kg.	1/25	2½ hrs.	2.75	7.5	
	1/26	24 "	2.91	8.1	
	1/31	6 days		10.0	

estimating calcium and the Briggs modification of the Bell-Doisy method for estimating the inorganic phosphorus. We did not at-

tempt to ascertain what ratio of the total calcium was diffusible or in an ionized state. To bring about this marked reduction of calcium, it sufficed to sever but one of the splanchnic nerves, and it did not seem to be of consequence whether this was the right or the left. The reduction in the calcium level occurred in about 24 hours, but the percentage was still low in some instances after an interval of 1 or 2 weeks. In all cases, however, it regained its normal level, evidently due to the action of a compensatory mechanism. The accompanying graph gives a typical picture of the course of the serum calcium following the severing of a splanchnic

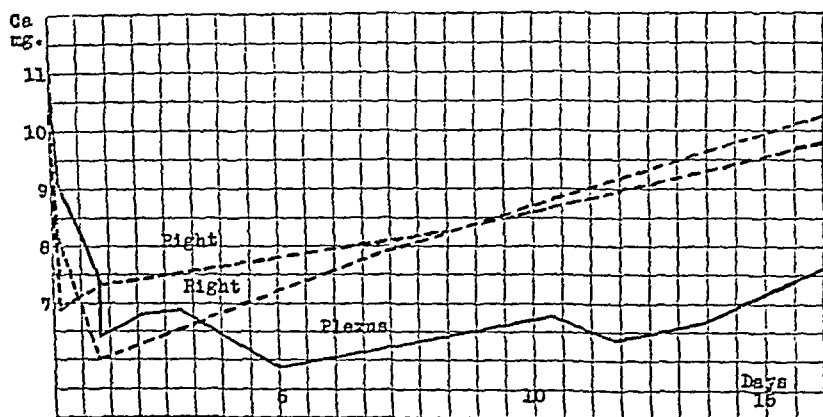


FIG. 1. Calcium of serum following section of the splanchnic nerves or plexus.

nerve. It should be added that the fall in calcium level cannot be attributed to the opening of the abdominal cavity or the handling of the viscera, as was proved by analyses of the blood of animals in which operations were performed to produce chronic intestinal obstruction and bile fistulae. Following abdominal operations on man it was found that there may be a slight fall in the calcium of the serum, a decrease of less than 1 mg. per cent; only a few estimations of this kind were made. It is perhaps worthy of note that recently Lebedenko (7) has described marked congestion and hemorrhages in the solar plexus following abdominal operations. It will be noted that the summary of operations reproduced in Table IV includes one in which the supra-

renal glands were decapsulated. This operative procedure was followed by a slight decrease in calcium 2 hours later, but nothing further was noted.

In contradistinction to the effect on calcium, the percentage of inorganic phosphorus in the blood, determined by the Bell-Doisy method, was found slightly changed, following the various lesions of the sympathetic nerves.

TABLE IV.

Summary of Operations on Sympathetic Nerves.

Dog		Area of sympathetic nerves severed	Serum Ca		Remarks
No.	Sex		Lowest level	Interval	
			<i>mg. per cent</i>		
8	♀	Few fibers of celiac plexus	8.2	1 day	
9	♂	Left splanchnic	7.5	2½ hrs.	
6	♀	Right "	6.0	25 "	
7	♂	" "	6.9	2½ "	
2	♀	Celiac plexus partially destroyed. Splanchnic cut (?)	8.4	3 "	Hemorrhage, followed by gangrene of intestines
4	♀	Celiac plexus partially destroyed	7.2	1 day	
3	♀	Celiac plexus partially destroyed	5.9	5 days	Low calcium over a 15 day period. No tetany
10	♂	Celiac plexus partially destroyed	6.7	1 day	
1	♀	Cervical sympathetic followed by right splanchnic and part of celiac plexus	6.1	3 days	
5	♂	Suprarenal glands decapsulated	9.6	2 hrs.	

Experiments on the Abdominal Vagi Nerves.

A series of experiments was next carried out to ascertain the effect of section of the vagi nerves on the calcium and phosphorus level of the blood. Under general anesthesia, through an upper median abdominal incision, both nerves were exposed as far cephalad as possible, just as they penetrated the diaphragm. In order to section the

nerves before the abdominal branches were given off traction downward was exerted. A segment of nerve 2 to 3 cm. in length was excised. This procedure had to be carried out with care in order to avoid pneumothorax.

There have been several investigations of the effect of vagotomy on the chemistry of the blood, but these studies have not considered calcium or phosphorus. Cokkalis (8) described a disturbance in the gas exchange of the lungs, an increase of 5 to 7 volumes per cent of the total CO₂ of the arterial blood. Papilian and

TABLE V.

Calcium and Inorganic Phosphate of the Blood Following Bilateral Abdominal Vagotomy.

Dog 11	Date	Interval after operation	Blood			Remarks
			Inor- ganic P	Serum Ca	Hemo- glob n	
	1927		mg	mg	percent	
Mongrel	6/14		3 2	11 3	85	Esophagus pulled down through diaphragm and both vagi cut; 3 cm. excised
♂		1½ hrs	8.4	11 0	87	
	6/15	18 "	4 2	10 7		Diet. bread and cooked meat, no milk
8 1 kg.		23 "	3 6	11 0	88	
	6/17	72 "	3 8	11 3		
	6/21	7 days	4 3	11 1	90	
	6/24	10 "	4 4	11 2		
	6/27	13 "	4 1	12.2		
	7/5	21 "	4 7	10 3		
	7/11	27 "	3 5	10 5		
	7/18	34 "	4 8	10 0		
	7/26	42 "	4 8	11 9		
	9/20	98 "	7 1	11 0		Blood sugar 58 9 mg. " " 93 8 "
	9/28	106 "	5 3	9 3		
	10/6	114 "	5 18	10 1		

Jianu (9) stimulated the sympathetic vagi nerves with suprarenin after section and found that the sugar was increased in both the arterial and the venous blood. Recently Frommel (10) severed the vagi nerves of rabbits and then tested the effect of calcium chloride on the heart; he could find no alteration.

Tables V, VI, and VII present in tabular form the results of three vagotomy experiments. The first shows a rise of calcium to 12.2 mg. 13 days after operation, the second a rise to 14.9 mg. after 8 days, and the third (Table VII) a rise to 13.6 mg. All of these figures are defi-

nately above the normal. In one instance there was a temporary fall of calcium about 24 hours after the vagi were cut (Table VI). It would seem that the conclusion may be drawn that this operation, in

TABLE VI.

Calcium and Inorganic Phosphate of the Blood Following Bilateral Abdominal Vagotomy.

Dog 12	Date	Interval after operation	Blood			Remarks
			Inor-ganic P	Serum Ca	Hemo-globin	
	1927		mg.	mg.	per cent	
♂	6/21		4.3	10.3	94	Previous to operation
13.2		1½ hrs.	4.9	10.8	91	Rapid, labored respiration for 15-20
kg.	6/22	19 "	3.9	11.1	90	minutes after recovery from anes-
		24 "	4.2	7.7	90	thesia
	6/23	48 "	4.0	9.1		
	6/24	72 "	3.7	10.7		Diet: bread and cooked meat
	6/27	8 days	4.4	14.9		
	7/5	16 "	5.35	11.2		
	7/11	22 "	5.23	10.85		
	7/18	29 "	4.45	10.8		
	9/20	93 "	4.89	10.4		
	9/28	101 "	4.90	10.57		Blood sugar 97.2 mg.
	10/6	109 "	5.32	11.0		" " 71.3 "

TABLE VII.

Calcium, Inorganic Phosphate, and Chloride of the Blood Following Bilateral Abdominal Vagotomy.

Dog 13	Date	Interval after operation	Blood				Remarks
			Inor-ganic P	Ca	Chlo-rides	Hemo-globin	
	1927		mg.	mg.	mg.	per cent	
♀	6/22		4.0	11.5	360	87	Diet of bread and cooked
Mongrel		1½ hrs.	2.5	11.4	356	85	meat
11 kg.	6/23	19 "	2.9	10.8	361		
	6/24	48 "	2.5	12.0			
	6/27	5 days	3.8	13.6	350		

contradistinction to severing of the splanchnic nerves, leads to an augmentation of the calcium of the blood. It is to be noted also that the equilibrium seems to have been definitely disturbed; we miss the con-

stancy in the calcium level which is such a characteristic feature in almost all normal mammals. In regard to the effect on inorganic phosphate the results are too irregular to warrant a definite conclusion. Attention, however, should be drawn to the sudden and temporary rise of phosphorus in one operation (Table V) from 3.2 to 8.4 mg. $1\frac{1}{2}$ hours after cutting the vagi. In general, it may be stated that this operation tended to bring about a slight disturbance in equilibrium of this ion.

DISCUSSION.

At the present time the physiologic and anatomic conceptions of the sympathetic nervous system are not identical. Whereas physiology has taken account but little of the afferent sympathetic fibers, anatomy has long since ascribed sensory fibers to the heart, mesentery, urinary bladder, etc. Moreover, until recently the centers in the spinal cord and midbrain were not regarded physiologically as a part of the sympathetic system. Nowadays, not only have these areas of the central nervous system been included, but the concept has been broadened functionally, so as to include some of the glands of internal secretion. In the present state of our knowledge it would be unwise to attempt to interpret the pathogenesis of the chemical changes which we have brought about by a destruction of these nerve paths. It would be of interest to know what has become of the calcium which is lacking in the blood in such marked degree. Has this deficiency come about from a failure of absorption, from an increased excretion by way of the bowel or the urinary tract, or does this loss represent merely an altered distribution in the tissues of the body? This question is of significance and we hope that further investigation will be able to shed light upon it. It cannot be ascribed to a mere alteration in the concentration of the blood for, as may be noted, percentages of hemoglobin before and after the various operations were but little altered. Whatever may be the physiologic mechanism involved, it would seem that in connection with the development of metabolic disturbances the rôle of the nervous system may well be of greater importance than is generally believed. In tetany, for example, we cannot take it for granted that nerve hyperirritability results solely from deficiency—either absolute or relative—of calcium, but must investigate whether the disturbance of nerve function may also be a primary factor.

CONCLUSIONS.

When a splanchnic nerve or the celiac plexus was severed a definite fall in serum calcium resulted, the level falling to 6 mg. per cent and remaining low for 1 or 2 weeks. Tetany, however, did not develop. The inorganic phosphate was slightly altered.

On the other hand, when the vagi nerves were severed, the serum calcium rose.

The equilibrium of both calcium and phosphorus was rendered less stable by the division of the sympathetic or the parasympathetic nerves.

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ALTERATIONS IN THE CALCIUM LEVEL OF THE BLOOD FOLLOWING SECTION OF THE SPINAL CORD.*

By ALFRED F. HESS, M.D., BENJAMIN N. BERG, M.D., AND ELIZABETH
SHERMAN.

*(From the Department of Pathology, College of Physicians and Surgeons, Columbia
University, New York.)*

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In connection with an investigation of the effect of nerve injury on the calcium and phosphorus content of the blood, it seemed of advantage to ascertain the effect of section of the spinal cord at various levels. As far as we know, a study of this kind has not been carried out.

It will be remembered that about 50 years ago Charcot aroused the medical world by a clinical description of lesions of the bones resulting from injury to the spinal cord—from pressure of carious vertebræ, tumors, etc., or associated with organic disease of the nervous system. Not only the bones but the tendons, ligaments, and the surrounding connective tissue were involved frequently in these pathologic changes. Since this time a variety of similar alterations have been described by clinicians and pathologists. In addition to lesions of the long bones, stone in the kidney has been noted from time to time in conjunction with fracture or injury of the spinal vertebræ. In 1895 Mueller (2) reported 10 cases of fracture of the spine, associated with prolonged paralysis, in which calculi were found in the kidneys upon postmortem examination; in many of these instances the compression was mild. Mueller believed that the calculi had developed as the result of a lesion of the nerves. However, as renal and bladder disturbances, as well as infection frequently accompany injuries to the spinal cord, it is difficult to judge whether these factors did not play the predominant rôle in the formation of the calculi. Some pertinent observations made in the course of the World War should be mentioned in this connection. It was found by army surgeons that wounds involving the spinal cord, resulting in paraplegia, were followed often by calcification of the joints and the surrounding tissues. By far the most complete report treating of this condition is that of the French investigator Ceillier (3) who published a monograph entitled "Para-osteo-arthropathies" in which he detailed 79 cases of

* Presented at the Annual Meeting of the American Society of Biological Chemists, Rochester, April 14, 1927 (1).

this kind, occurring among 160 paraplegics. The cases which resulted from bullet wounds, shrapnel, bombs, falls, etc. were studied by means of radiographs. Calcification resulted apparently irrespective of the level of the cord lesions; the highest injury was at the second dorsal vertebra. In all instances the lesion was severe, leading to complete or almost complete paralysis and loss of sensation in the extremities. Similar, but less convincing observations were published from the records of the British Army. More recently Meyer (4) has reported an interesting case of compression fracture at the eleventh dorsal vertebra with contusion of the upper lumbar cord. Six months later symmetrical formation of bone developed in the connective tissue of both quadriceps muscles and the adjacent fascia and periosteal tissue, and 10 months after the injury large calculi were found in both kidneys.

EXPERIMENTS.

Dogs were placed under complete ether narcosis and a laminectomy was carried out in the typical way, and the cord transected at the desired level. The technique was not complicated and the animals withstood the operations well. Following transection, the rent into the dura was sutured in order to avoid leakage of spinal fluid. The level at which the incision was made was checked at postmortem examination and at times found to have been slightly higher or lower than had been anticipated. The bleeding was moderate. A flaccid paralysis of both hind legs came about rapidly and was associated at times with a transient spasticity of the fore legs. The respiration and swallowing movements remained unaltered and nourishment was well taken. Precautions were taken against chilling. The animals were bled from the jugular vein before operation and as a rule 2 hours subsequently and at varying intervals during the next few days.

The results of typical successful operations are given in Tables I and II. The first shows that the percentage of calcium in the serum was 9.6 mg. previous to operation, rising to 17 mg. 22 hours later, and was 16.4 mg. 46 hours subsequent to the operation. The Kramer-Tisdall method was employed for estimations of calcium and the Briggs modification of the Bell-Doisy method for inorganic phosphorus. At necropsy the cord was found completely severed between the first and second dorsal segments except for a few fibers anteriorly. A similar result was obtained in the operation, of which the details are summarized in Table II. In this instance the calcium content rose to 13.2 mg. 2 hours and 15 mg. 21 hours subsequent to division of the cord. It will be noted that the equilibrium of the calcium was also markedly disturbed, that for some days it fell even below the normal level but that gradually it regained

the normal percentage of between 9 to 10 mg. It must be added, however, that these striking chemical changes could not be brought about at will. In spite of the fact that section of the cord was made in three other animals apparently at the same plane, little or no fall in the percentage of calcium resulted. The probable explanation for this irregularity is that although the operative procedure was seemingly identical, the lesion of the nerve cells was essentially different. Experiences of this kind are not uncommon in the course of experimental surgery on the spinal cord and brain.

TABLE I.

Calcium and Inorganic Phosphate of the Blood Following Transection of Spinal Cord between First and Second Dorsal Segments.

Dog 1	Date	Interval after operation	Blood		Remarks
			Inorganic P	Serum Ca	
	1927	hrs	mg.	mg.	
Mongrel ♂	2/10		3 62	9 6	Previous to operation
		2	2 98	9 6	Good recovery. Complete flaccid paralysis below level of lesion
9 2 kg.	2/11	22	3 35	17.0	Milk feeding
	2/11	27	3 40	10 0	
	2/12	46		16.41	
	2/14				Died. Autopsy showed infection of wound; cerebrospinal fluid clear. A few anterior fibers of cord not severed. Parathyroid glands normal macroscopically and microscopically

Noting that Ceillier attributed the calcification in his cases to an injury of the sympathetic nerves supplying the blood vessels and to a consequent edema and hyperemia, we made an attempt to reproduce the effect of the bullet and shrapnel wounds. With this in view the cord was traumatized at different levels below or in the neighborhood of the incision. This procedure, which was carried out with the animals under complete anesthesia, was without effect.

In other instances, the spinal fluid was allowed to escape freely from the canal and in still others the nerve roots were resected in addition to cutting the spinal cord. For example, in one operation where the

cord was divided between the first and second dorsal segments, the nerve roots of the first, second, and third dorsal areas were divided likewise on both sides. A similar procedure was carried out in connection with the division of the cord between the first and second lumbar segments, as well as at the eighth cervical segment. It seems unnecessary to record the percentages of calcium in these various

TABLE II.

Calcium and Inorganic Phosphate of the Blood Following Transection of Spinal Cord between First and Second Dorsal Segments.

Dog 2	Date	Interval after opera- tion	Blood		Remarks
			Inor- ganic P	Serum Ca	
	1927	hrs.	mg.	mg.	
♂	2/15		3.46	9.9	Previous to operation
8.8 kg.	2/15	2	3.60	13.2	Good recovery. Complete flaccid paralysis below level of lesion. Incontinence of urine
	2/16	21	3.44	15.0	Milk 100 cc.
		26		11.1	
	2/17	44	4.02	6.1	Wound infected. Milk 500 cc.
		52	3.86	10.1	
	2/18	74	3.94	7.9	Condition good. Pulse 125, respirations 14. Milk 600 cc., bread 10 to 20 gm.
		days			
	2/19	4		11.0	Blood CO ₂ 49.95. Condition good. Pulse 116, respirations 60, temperature 38°. Reflexes hyperactive. Milk 400 cc.
	2/20	5		9.6	Pulse 120, respirations 70, temperature 38.2°. Milk 400 cc.
	2/21	6	3.78	9.8	Temperature 38°, respirations 22. Milk 250 cc.
	2/22	7		9.0	Killed. Wound infected. No meningitis. No pneumonia

operations. The results can be summarized by the statement that there was no significant change.

In two instances the cord was divided in two places. In one animal section was made first at the upper dorsal segment and some days later at the seventh cervical level, and in another it was cut first between the seventh and eighth cervical segments and 2 days later at the

third dorsal level. In neither case was the calcium level altered significantly. In fact, among a total of fourteen operations on the cord at various levels, definite and striking changes could be brought about only following section at the upper dorsal segments.

It was thought that the disturbance in the calcium might be due to an effect on the parathyroid glands through the nerves which regulate their activity. The nerve supply of the parathyroids has not been worked out with the same completeness as that of the thyroid gland. In order to test this hypothesis the thyroid and parathyroid glands were removed to observe whether the diminution of calcium which is

TABLE III.

Thyroparathyroidectomy Followed by Transection of Spinal Cord at Third Dorsal Segment.

Dog 3	Date	Interval after operation	Blood		Remarks
			Inor- ganic P	Serum Ca	
	1927	hrs.	mg.	mg.	
Mongrel ♀ 12 kg.	4/19		2.86	10.1	<i>Thyroidparathyroidectomy</i>
		2½		8.2	
		5		7.8	
	4/20	22½	2.74	8.0	Pulse 80, respirations 60, temperature 38.5° Pulse 110, respirations 80, temperature 38.5°.
					No tetany. Sensitive to noises
	4/20	24			<i>Section of spinal cord</i>
		1	3.52	11.1	Hoarse barking. Respirations 35 to 72
		5	3.51	6.7	Rapid shallow breathing. 400 cc. water
	4/21	21		6.2	500 cc. water. Killed

brought about by this means could be raised by subsequent division of the spinal cord at the upper dorsal segment. Table III shows the result of this procedure. It will be noted that 5 hours subsequent to parathyroidectomy the serum calcium had fallen from 10.1 to 7.8 mg. per cent, and a condition of latent tetany had developed. The following day the cord was sectioned at the third dorsal segment. One hour later the calcium had risen to somewhat above the original percentage, namely to 11.1 mg. In other words, the rise in calcium which followed division of the spinal cord was not to be ascribed to a stimulative effect on the parathyroid glands and appeared quite independent of them.

It is impossible at present to explain the marked alteration in the percentage of calcium resulting from section of the spinal cord. The only procedure which is known to produce a similar effect is the injection of parathyroid extract. It would seem as if the alteration must be due to a disturbance of the sympathetic system. The anatomy of these nerves and centers within the spinal cord has not, however, been determined with sufficient accuracy to enable one to estimate the validity of this interpretation. Some years ago Biedl (5), in a paper on the splanchnic centers, described spinal centers of the sympathetic at the boundary between the cervical and dorsal cord, concentrated mainly between the eighth cervical and second dorsal but extending down even to the fifth dorsal segment. These cells varied in their sites within the cord at different levels. More recent anatomic studies of the spinal centers of the vegetative nervous system have served to locate them in the intermediary lateral tract of the cord. According to Mueller (6), who recently has reviewed this subject, no studies of these cells have been carried out in cases of tabes or of syringomyelia. It would seem that at the present time little can be gained by bringing forward further hypotheses in connection with the phenomenon which we have described.

CONCLUSIONS.

A marked rise and disturbance in equilibrium of the calcium in the blood was brought about by section of the spinal cord in the upper dorsal segment. This reaction, however, was not constant.

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RECENT STUDIES ON METHODS OF ISOLATING A BACTERIOPHAGE FOR *BACILLUS DIPHTHERIÆ*.

BY JULIUS A. KLOSTERMAN AND KATHRYN W. SMALL, M.D.

(From the Laboratories of the Department of Bacteriology, College of Dentistry,
New York University, New York.)

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D'Hérelle, Fejgin and Blair (1-3) have published accounts of their success in isolating a lytic principle for *B. diphtheriæ*. In the light of increasing interest in studies of bacteriophagy in general, it appears advisable to contribute to the available literature additional experimental data pertaining to the isolation and activity of a true bacteriophage for *B. diphtheriæ*.

The writers have exercised particular care to utilize only toxic strains of *B. diphtheriæ* throughout their entire investigation. The purpose was to secure a phage active against virulent strains, to be used in proposed subsequent experiments.

Unfortunately, d'Hérelle has given insufficient information concerning the details of his investigation on antidiphtheria bacteriophages. Hence duplication of his researches for corroboration could not be attempted. However, the two phages which he reported to have isolated from the feces of diphtheria antitoxin horses were active only upon atoxic strains of *B. diphtheriæ*. The mode of detecting the bacteriophagic activity was not disclosed in the instance of this particular phage.

Fejgin briefly describes having isolated a diphtheria phage from a Martin bouillon culture of *B. diphtheriæ* several weeks old. In an effort to demonstrate the activity of this phage on agar, she secured but a single plaque, in the center of which one bacterial colony subsequently developed. Unfortunately she was unable to proceed with her investigation as the tube was broken in handling.

Blair, on the other hand, reported isolations of antidiphtheria phages with comparative ease from three distinct sources. He based these findings entirely upon the degree of clearness of broth cultures containing the filtrate to be tested for phage, as compared to the turbidity of the broth culture controls. In no instance

has he included accounts of antidiphtheria phage activity on agar. The latter characteristic, according to the most recently developed technical procedure, appears to be absolutely necessary in establishing the fact of true bacteriophagy. Lytic action based wholly upon apparent lysis in broth cultures may be due, particularly if not capable of serial transfer, to inhibitory or enzymatic substances which by chance are present in the suspected filtrates rather than to a real bacteriophage. Another extremely important factor which also decides the genuineness of a bacteriophage is the well known characteristic of cultivation by successive transferring with the microorganism for which it is active. Blair gave no indication that his phages were propagatable.

The following protocols outline the procedure used in the attempt to isolate an antidiphtheria phage. Four cardinal principles were rigidly adhered to—first, the use of only definitely toxic strains, second, requiring all suspected phages to show activity on agar slants as well as in broth, third, carrying all tests on filtrates through two or more transfers before discarding because of lack of visible phage action, fourth, requiring that any suspected phage be capable of propagation by subculturing in the presence of a toxic strain of *B. diphtheriæ*. Fecal material collected from either human or animal sources was treated as follows. Approximately a cc. of feces was placed in 20 cc. of broth (pH 8.2) and allowed to incubate overnight at 37°C. It was then filtered through infusorial earth packed in a Buchner funnel. The filtrate was finally passed through a Berkefeld filter (*w*).

The first attempt at isolation was made by using the feces of an active case of diphtheria.¹ The infecting organism was isolated from the throat of this patient and was made use of in the following procedure. It is referred to as the homologous strain. Eleven stools were examined. The first specimen was taken on the 5th day of illness and the others at intervals for the next 2 weeks covering course and convalescence. Of the eleven stool filtrates eight were tested upon one homologous and two heterologous strains. Two were tested upon one homologous and five heterologous strains. One was tested upon one homologous and six heterologous strains. In no instance was there any indication of the presence of a phage.

Next a 33 day old culture in 30 per cent serum broth of a recently isolated virulent strain of *B. diphtheriæ* (Strain A) was filtered and used

¹ The authors wish to thank Dr. W. H. Park for his kindness in making arrangements to secure the materials used in these experiments.

as a potential source of a phage. It was tested against the seven strains of *B. diphtheriae* which included its homologous strain and gave entirely negative results.

The third source tested for the presence of an antidiphtheria phage was the intestinal contents and peritoneal washings of guinea pigs inoculated with diphtheria cultures (Tables I and II).

TABLE I.
Test Animals.

Showing animal inoculations, time of death, result at autopsy with respect to signs of diphtheria toxemia and result of test for phage activity on intestinal contents and peritoneal washings.

Infecting strain used	Broth suspension, 1 cc.										Broth culture, 1 cc.				
	Intraperitoneal						Subcutaneous				Subcutaneous				
	Guinea pig No.	Death	Autopsy findings	Phage activity		Guinea pig No.	Death	Autopsy findings	Phage activity		Guinea pig No.	Death	Autopsy findings	Phage activity	
				Intestinal contents	Peritoneal washings				Intestinal contents	Peritoneal washings				Intestinal contents	Peritoneal washings
		hrs.					hrs.					hrs.			
<i>B. diphtheriae</i> Strain M 1314	1	48	+	-	-	4	48	+	-	-	6	48	+	-	-
<i>B. diphtheriae</i> Strain A	2	24	+	-	-	5	48	+	-	-	7	Killed at 48	-	-	-
<i>B. diphtheriae</i> Strain F	3	48	+	-	-						8	48	+	-	-
			(also peri- toneal abscess)												

Eleven animals were used of which eight served as test pigs and three as controls. Three toxic strains of *B. diphtheriae* were employed, namely Strain M 1314, Strain A and Strain F. The first two had been isolated 7 months ago while the third was isolated 3 weeks previously. Three different types of inoculations were made in the case of two strains, while only two of these were employed with the third strain. Hence altogether eight guinea pigs were used as test animals. The types of inoculations resorted to were first, 1 cc. of a suspension made by emulsifying the bacterial mass of an 18 hour serum agar slant in 5 cc. of broth, adminis-

tered intraperitoneally; second, the same material administered subcutaneously and third, 1 cc. of a 48 hour broth culture given subcutaneously.

One control pig was used for each of the three strains of *B. diphtheriæ*. In each case the pig received a subcutaneous inoculation of 1 cc. of a 48 hour broth culture of its assigned strain and in addition two hundred units of diphtheria antitoxin given intraperitoneally. These controls served as an indirect check on the toxin-producing property of the listed strains. In other words they were included for the purpose of demonstrating that all of the strains used produced toxin which was neutralized by a specific antitoxin.

The results were as follows. One test animal (No. 2) receiving an intraperitoneal inoculation of a suspension of Strain *A* died in 24 hours. The autopsy revealed signs of diphtheria toxemia which were the presence of a serous exudate in the peritoneal cavity, congestion of the peritoneum and enlargement and congestion of the adrenals. Six other test pigs (Nos. 1, 3, 4, 5, 6, 8) died in 48 hours with

TABLE II.

Control Animals.

Showing animal inoculations used to control specificity of toxin produced by listed strains.

Infecting strain used	Diphtheria antitoxin administered subcutaneously	Guinea pig No.	Broth culture (subcutaneous)	
			Death	Autopsy findings
<i>B. diphtheriæ</i> Strain <i>M</i> 1314	200 units	9	11 days	Cause of death undetermined (no signs of diphtheria toxemia)
<i>B. diphtheriæ</i> Strain <i>A</i>	200 units	10	4 days	Streptococcus infection (no signs of diphtheria toxemia)
<i>B. diphtheriæ</i> Strain <i>F</i>	200 units	11	Alive	

positive signs at autopsy. The remaining test pig (No. 7) having received a subcutaneous broth inoculation of Strain *A* was killed and autopsied with negative results, however.

One control (No. 11) was alive on the 20th day. A second control (No. 10) died on the 4th day. Before inoculation the latter pig showed a fluctuating swelling of the perineal region. At autopsy there were no signs of diphtheria toxemia. However, there were soft swollen inguinal and retroperitoneal lymph nodes (bilateral) and an enlarged, congested spleen. Smears from the lymph nodes showed many Gram-positive cocci in long chains. Hence the animal probably succumbed to an acute streptococcus infection, the focus of which was the lesion observed before inoculation. The third control (No. 9) died on the 11th day and autopsy failed to reveal the cause of death but certainly there was no indication that it was due to diphtheria toxemia.

At autopsy one of the intraperitoneal suspension pigs (No. 3) showed a small peritoneal abscess at the site of inoculation. A smear and culture from the lesion showed *B. diphtheriæ*. The abscess and surrounding wall were emulsified in broth, incubated and filtered.

This filtrate and those made from intestinal contents and from peritoneal washings of the eight pigs which received culture without antitoxin were examined

TABLE III.

Results of phage activity of filtrates of feces from antitoxin horses on strains of *B. diphtheriæ*.

Results indicated are those recorded after two transfers.

Strains of <i>B. diphtheriæ</i>	Filtrates from feces of antitoxin horses					
	149	152	161	87	143	144
<i>B</i>	—	—	—	—	—	—
<i>C</i>				—	—	—
<i>D</i>				—	—	—
<i>E</i>				—	—	—
Park 8	—	—	—	—	—	—
M 1314	—	—	—	—	—	—

Blank space indicates these combinations were not tested.

TABLE IV.

Results of phage activity of filtrates of feces of five additional antitoxin horses on seven strains of *B. diphtheriæ*.

Results indicated are those recorded after two transfers.

Strains of <i>B. diphtheriæ</i>	Filtrates from feces of antitoxin horses				
	165	142	156	136	151
<i>A</i>	—	—	—	—	—
<i>B</i>	—	—	—	—	—
<i>C</i>	—	—	—	—	—
<i>D</i>	—	—	—	—	—
<i>E</i>	—	—	—	—	—
Park 8	—	—	—	—	—
M 1314	—	—	+	—	—

for the presence of phage. Each filtrate was tested on its homologous strain and gave negative results. Then all filtrates were tested on two heterologous strains of *B. diphtheriæ* and six of the filtrates were additionally tried on six other heterologous strains of *B. diphtheriæ*. All results were negative (Table I).

The final attempt to isolate an antidiphtheria phage was conducted by using the filtrates of feces emulsions of antitoxin horses. The feces of six horses² retained at Otisville (for the New York City Department of Health) for the production of diphtheria antitoxin were emulsified, incubated and filtered according to the usual procedure. Of these six filtrates three were tested on three strains of diphtheria and three were tried on six strains. As the toxin of Strain Park 8 is used for the stimulation of antitoxin in these horses, it is regarded as the homologous strain. All efforts to isolate a phage from these filtrates failed (Table III).

TABLE V.

Observations on specificity of two generations of antidiphtheria Phage 156 *M* 1314 on Strains of *B. diphtheria*.

Results indicated are those recorded after two transfers.

Strains of <i>B. diphtheria</i>	Antidiphtheria Phage 156 <i>M</i> 1314 Generation 11	Antidiphtheria Phage 156 <i>M</i> 1314 Generation 23
<i>A</i>	—	—
<i>B</i>	—	—
<i>C</i>	—	—
<i>D</i>	—	—
<i>E</i>	—	—
<i>F</i>		+
<i>G</i>		+
Park 8	—	—
<i>M</i> 1314	+	+

Blank space indicates these combinations were not tested.

Filtrates from five other horses immunized with diphtheria toxin showed activity against one of these strains. This is the only instance in which the authors secured an antidiphtheria phage (Table IV).

The filtrate of Horse 156 showed lysis of *M* 1314 in broth and on agar in the second generation. The test broth was only slightly less turbid than the control and on agar phage activity was evident only in the form of isolated plaques. The fourth generation showed confluent plaques on the agar. The successive generations in broth gradually showed more and more lysis but even in the twenty-second

² The toxin used to immunize these horses consisted of the supernatant fluid of broth cultures which were allowed to sediment.

generation lysis is not complete. 500 units of concentrated diphtheria antitoxin were mixed with culture and phage in broth to counteract any undesirable action of toxin upon phage activity. There was

TABLE VI.

Observations on specificity of antidiphtheria Phage 156 *M* 1314 tested on eighteen strains of microorganisms other than toxic *B. diphtheriæ*.

Results indicated are those recorded after two transfers.

Microorganisms	Antidiphtheria Phage 156 <i>M</i> 1314 (Generation 16)
Diphtheria-like Strain 1154 (non-toxic)	—
Diphtheria-like Strain 1175 (non-toxic)	—
Diphtheria-like Strain 1178 (non-toxic)	—
Diphtheria-like Strain <i>U B</i> (non-toxic)	—
<i>B. xerosis</i>	—
<i>B. hoffmanni</i>	—
<i>Staphylococcus aureus</i>	—
<i>Staphylococcus albus</i>	—
<i>Staphylococcus citreus</i>	—
<i>B. coli communior</i>	—
<i>B. dysenteriæ</i> Shiga	—
<i>B. dysenteriæ</i> Flexner	—
<i>B. dysenteriæ</i> Mt. Desert	—
<i>B. typhosus</i> (Pfeiffer)	—
<i>B. paratyphosus</i> A	—
<i>B. paratyphosus</i> B	—
Cholera-like	—
<i>B. subtilis</i>	—

TABLE VII.

Observations on thermal inactivation point of antidiphtheria Phage 156 *M* 1314

Temperature	30 min exposure	45 min exposure
°C		
50	Active	Active
55	—	—
60	—	—

no apparent difference in end-result between the test containing antitoxin and the control without antitoxin as far as lysis due to phage activity was concerned.

Two later specimens of feces from Horse 156 were tested for the presence of phage and were found negative.

The blood serum of Horse 156 whose first specimen of feces gave positive results on *M* 1314 was tested on this culture but failed to give evidence of a phage.

The specificity of Phage 156 *M* 1314 was studied. The eleventh generation was tested on six diphtheria strains other than Strain *M*

TABLE VIII.

Results of using non-diphtheria phages in conjunction with *B. diphtheriae* Strain *M* 1314 for determining whether or not these known bacteriophages are active on this strain.

Results indicated are those recorded after two transfers.

Name of phage	Source of phage	Organism on which phage is active	Generation of phage	Activity on <i>B. diphtheriae</i> Strain <i>M</i> 1314
Coli	Not recorded*	<i>B. dysenteriae</i> Mt. Desert	10	—
Coli	East River water	<i>B. coli communior</i>	5	—
Coli	Bellevue sewer water	<i>B. coli communior</i>	5	—
Coli	Willard Parker sewer water	<i>B. coli communior</i>	7	—
Coli	Normal cat feces	<i>B. coli communior</i>	8	—
Dysentery	Feces from case of typhoid	<i>B. dysenteriae</i> Shiga	5	—
Dysentery	Sample from pail of milk just collected by farm hand	<i>B. dysenteriae</i> Shiga	9	—
Dysentery	Human saliva pooled from six sources	<i>B. dysenteriae</i> Shiga	12	—
Typhoid	Feces from case of dysentery	<i>B. typhosus</i> (Pfeiffer)	6	—

* This phage was supplied through the courtesy of Dr. J. J. Bronfenbrenner to whom the authors are also indebted for valuable suggestions and for his kindness in reviewing this paper.

1314, with negative results. The twenty-third generation was tested on the same six strains and two additional ones. It was active against the latter two. These two strains, namely Strain *F* and Strain *G* as well as Strain *M* 1314, were all recently isolated; that is within 6 months (Table V).

The sixteenth generation of antidiphtheria Phage 156 *M* 1314 was tested on eighteen different strains of microorganisms other than toxic *B. diphtheriae* with negative results (Table VI).

The thermal inactivation point of Generation 23 of antidiphtheria Phage 156 *M* 1314 was found to lie between 50°C. and 55°C. (Table VII).

Generation 22 was titrated by a modified broth dilution method (4). In carrying out the final transfer, however, the authors substituted filtration for heating as this phage is inactivated at a comparatively low temperature. The phage showed activity when it was present in a 1×10^{-9} dilution.

Nine non-diphtheria bacteriophages of high titer previously isolated from various sources were tested for the purpose of determining whether or not they were active on *B. diphtheriæ* Strain *M* 1314 (Table VIII).

SUMMARY.

Of the attempts to isolate an antidiphtheria phage (1) from stools collected daily during the course of a case of the disease, (2) from a 33 day old broth culture of *B. diphtheriæ*, (3) from intestinal contents and peritoneal washings of guinea pigs inoculated with three different toxic strains of *B. diphtheriæ*, none yielded an antidiphtheria phage. However of eleven specimens of feces collected from eleven antitoxin horses one was found to contain a bacteriophage active against *B. diphtheriæ*. This phage was not observed in the first generation and did not show up until transferred the second time. Had the results of the first transfer been regarded as final in all certainty the existence of a phage would not have been recognized. Two additional specimens of feces from the positive horse (No. 156) were later tested to determine whether this bacteriophage was continually present in the intestinal tract of this animal. Both of these feces filtrates failed to yield a phage. Later a sample of freshly collected blood serum from the same horse was tested and found not to contain a phage.

The antidiphtheria phage was tested against eighteen non-diphtheria strains of microorganisms to determine whether it would show lytic activity for related or unrelated bacteria. There was no evidence of lysis in any of these types. The specificity of this phage was also tested on nine strains of *B. diphtheriæ* and found to be active on three heterologous strains (Strain *M* 1314, Strain *F* and Strain *G*). These incidentally were all recently isolated. It failed to lyse the remaining six strains of which five were recently isolated.

B. diphtheriae Strain *M* 1314 was used in combination with nine heterologous bacteriophages isolated from various sources, to determine if any of these phages would by chance lyse this culture. The results were all negative.

To date this phage fails to show complete lysis although the twenty-second generation has a titer of 1×10^{-9} .

Additional proposed experiments involving this phage are being planned and will be undertaken in the near future.

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THE INFLUENCE OF HEATING THE IMMUNIZATION MATERIAL UPON THE ANTIBODY-INVOKING EFFECTIVENESS OF THE TYPE-SPECIFIC AND SPECIES-SPECIFIC ANTIGENS OF TYPE II PNEUMOCOCCUS CELLS.

By EMIDIO L. GASPARI,* JOHN Y. SUGG, WILLIAM L. FLEMING, AND
JAMES M. NEILL, Ph.D.

(From the Department of Bacteriology and Immunology, Vanderbilt University Medical School, Nashville.)

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INTRODUCTION.

Avery and Heidelberger and their associates (1-4) have presented convincing evidence that the virulent pneumococcus cell contains two different types of antigens: (1) a type-specific antigen (SP) which gives rise to a type-specific antibody; (2) a species-specific antigen (P)¹ which gives rise to a species-specific antibody. The type-specific antigenic complex is contained in the intact cell of strains possessing the soluble specific substance; immunization of a suitable animal with suspensions of pneumococcal cells invokes a type-specific antibody which reacts specifically with the soluble specific substance whether contained in solution (S-precipitation) or disposed on the periphery of the bacterial cell (agglutination); the same antibody is also apparently the one responsible for the passive protection of mice, at least against Type II pneumococci (5). The species-specific antigen, in contrast to the type-specific one, gives rise to an antibody

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¹ The term species-specific "antigen" is used in the inclusive sense of Avery and Heidelberger (1, 2) to refer to the protein (mainly nucleoprotein and mucoid) which reacts with the species-specific precipitins that are invoked by immunization with protein derived from all types of pneumococci. It is not important in this paper that the protein may include a number of separate but unrecognized antigens, provided they are sufficiently alike in chemical and physical properties to be affected in similar manner by heat.

reactive with the protein derived from all types of pneumococci. This antibody is especially prominent in the immune serum obtained by the injection of solutions of the pneumococcus nucleoprotein (3) or of filtered solutions of the endocellular substances which are liberated when pneumococci are dissolved by bile or disintegrated by freezing and thawing (4). The prominence of these species-specific antiprotein precipitins in the immune serum invoked by solutions of pneumococci is due to the absence of the type-specific antibody which is dominant in the usual antibacterial serum obtained by injection of the intact cells. However, certain amounts of the species-specific antibody are also present although seldom so prominent, in the type-specific immune serum.

Although both anti-S and anti-P occur together in the usual antibacterial serum, sufficient evidence (1-4, 6) has been presented to prove that each of the antibodies is distinct and separate and that each is invoked by a distinct and separate antigen. The present investigation proceeds upon that basis, and consists of a comparison of the effect of heating of the immunization material (suspensions of pneumococci) upon the antibody-invoking property of the type-specific (SP) and species-specific (P) antigens of Type II pneumococcus cells.

Any comparison of the influence of heat upon the antibody-invoking effectiveness of antigens should take into account the size of the doses injected into the animals, the total amount of antigenic material, number of injections or stimuli, and the length of time of the immunization. Although these factors are frequently ignored, it is obvious that comparisons of the effectiveness of unheated antigen with heated antigen are of little value if the dosages of total antigen are greatly in excess of that required for the maximum immunity response of the animal (7). All these factors were carefully controlled in our experiments; the animals were immunized with two different dosages and the sera tested at different stages of the immunization. While individual differences are to be expected, and were encountered, in the degree of responses of the individual rabbits, a sufficient number of rabbits (41) were included to furnish a valid basis for a comparison of the influence of heat upon the effectiveness of the antibody-invoking property of the type-specific and species-specific antigenic constituents of Type II pneumococci.

In one sense, the species-specific antigen (P) can be considered an endocellular antigen contained inside of the type-specific pneumococcal cell. Since these bacteria autolyze readily, the suspensions of pneumococci employed in routine immunizations contain significant amounts of free intracellular substances in solution; and, as pointed out by Avery and Heidelberger (2), animals injected with such material receive, in fact, a mixture of varying proportions of free or dissociated P together with the undissociated type-specific antigenic complex. This free P can be expected to give rise to species-specific antibody and thus the amount of autolysis which has occurred *in vitro* before injection of the material is certainly a factor in determining the species-specific antibody response. Precautions to avoid the presence of autolytic products were especially necessary with the unheated bacteria which autolyze more rapidly *in vitro* than do the heated cells, and for this reason fresh bacterial suspensions were prepared at least every 2nd day. However, in spite of all precautions, it is doubtful whether it is possible to obtain a suspension of pneumococcus cells, either heated or unheated, in which no cellular changes (freeing of the P) have occurred. Since dissolution of pneumococci takes place in the animal body, *in vivo* lysis should also be considered a possible source of the species-specific antigen when animals are immunized with suspensions of pneumococcus cells (2). In the present investigation, both possible sources of the species-specific antigen are considered since both filtered solutions of intracellular substances and vaccines with the minimum of autolysis were employed as immunization material.

EXPERIMENTAL.

Tests of the Immune Sera.—The immune sera were tested for type-specific and species-specific antibodies by the same general methods as those employed at the Hospital of The Rockefeller Institute (6). The type-specific antibody (anti-S) was recognized by three methods: (1) precipitation of the soluble substance from solution; (2) type-specific agglutination of broth cultures of the homologous strain; (3) passive protection of mice (8). The precipitation of the soluble substance (S) was tested with filtrates of young, unautolyzed broth cultures of pneumococci; the substitution of these fluids for solutions of the purified S substance being justifiable since they were devoid of the species-specific protein and contained no other detectable serologically reactive substance other than the type-specific S which is liberated in the early growth of pneumococci. The tests for the species-specific antiprotein precipitin were made with a filtered solution of Type I pneumococcus cells in which the species-specific antigen is the only substance reactive with Type II immune serum.

Experiment 1. Influence of Heat upon the Antibody-Involving Properties of the Protein Contained in Solutions of Pneumococcus Cells.

Solutions of pneumococcus cells lack entirely the effective antigenic complex which is responsible for the production of the type-specific antibody invoked

by suspensions of intact pneumococcus cells and hence the dominant antigen contained in the bacterial solutions is the species-specific protein (P) which gives rise to anti-P precipitins. Although our primary interest was in a study of the conditions determining the relative proportion of the two types of antibodies (anti-S and anti-P) invoked by the intact bacterial cells (vaccine), the first step in the investigation consisted of a study of the influence of heat upon the antibody-invoking property of the protein as contained in the cellular solutions. Previous experiments (9) had shown that the anti-protein precipitin response was slightly diminished by heating pneumococcus solutions for 10 minutes at 55°C. and greatly decreased by boiling. In the following experiment, the bacterial solutions were heated for 30 minutes at 55°, 75°, 100°, and 120°C., and the antibody-invoking activity of the heated solutions was compared with that of unheated pneumococcus solutions. In order to make the comparison more valid, two test doses were employed, one of which was 20 times as great as the other; the use of the two dosages furnishing a basis to compare the antigenic effectiveness of small amounts of the unheated solution with the effectiveness of large doses of the heated material. Test bleedings were made after two and after five courses of injections; the examination of the sera after two different periods of immunization furnishing a basis for comparison of the effectiveness of unheated and heated antigen from the standpoint of the relations of the total amount of antigen and of the number of injections required to invoke comparable immunity responses. The results are summarized in Table I.

The results of this experiment (Table I) showed that heating for 30 minutes at any temperature between 55° and 120°C. reduces the antibody-invoking activity of the species-specific protein contained in solutions of pneumococcus cells. Two courses of injections with a small dose (0.1 cc.) of the unheated solution invoked a more effective response than was obtained by immunization with the heated material, not only when the doses of unheated and heated material were equal but also when the dosage of heated material was 20 times as great as that of the unheated antigen solution. The same effect was evident when the immunization was continued to a total of five courses of injections: although the prolonged immunization with heated solution caused some increase in the anti-P precipitin content of the sera, the antibody responses to the heated antigen were always smaller and approached that invoked by the unheated material only in animals which had received doses 20 times as large as those required when the unheated solution was used. Thus, a comparison of the response to the unheated pneumococcus solution with the response to the heated solutions, shows that 30 minutes exposure to temperatures from 55°

to 120°C. diminished the effectiveness of the species-specific protein antigen, both from the standpoint of the degree of antibody response to equal doses of unheated and of heated solution, and also from the standpoint of the amount of total antigen (dosage) and number of injections required for an effective response.

TABLE I.

Influence of Heat upon the Antibody-Invoking Properties of Solutions of Pneumococcus Cells.

Antigen used in immunization: Pneumococcus cell solution		Species-specific protein precipitins (anti-P)				Type-specific antibodies (anti-S)
		Serum after 2 courses of injection		Serum after 5 courses of injection		Serum after 5 courses of injections
Treatment of antigen	Size of dose injected	Protein solution				Agglutinins Anti-S precipitins Passive protection
		Undiluted	1/10 diluted	Undiluted	1/10 diluted	
	cc.					
Unheated	0.1	++	+	+++	++	0
Heated 30 min. at 55°C.	0.1	±	0	+	±	0
	2.0	+	±	++	+	0
Heated 30 min. at 75°C.	0.1	0	0	±	0	0
	2.0	+	+	++	+	0
Heated 30 min. at 100°C.	0.1	0	0	±	0	0
	2.0	+	±	+	±	0
Heated 30 min. at 120°C.	0.1	0	0	0	0	0
	2.0	±	0	±	0	0

0 = no detectable reaction; ± = faint cloudiness without sedimentation; + = cloudiness with slight sedimentation; ++ = marked cloudiness with moderate sedimentation; +++ = marked cloudiness with large amounts of sediment.

A comparison of the responses to equal doses of the solutions which had been heated at different temperatures shows a more or less gradual diminution of the antibody-invoking property with increase in the temperature. The solution heated for 30 minutes at 55° or at 75°C. induced somewhat better responses than did the solution heated at

100°C. but the difference is less than that between the unheated solution and the solution heated at any of the test temperatures. While heating at 120°C. almost completely destroyed the antigenicity, prolonged immunization with the larger dose (2.0 cc.) invoked a weak anti-protein response.

The retention of some degree of antigenicity by the P substance of *Pneumococcus* after 30 minutes exposure to 120°C. is especially interesting in view of the relatively large loss in activity which follows heating at only 55°C. The antigenic effectiveness of coagulated proteins (9) involves fundamental questions which are encountered in the study of enzymes as well as in immunology, *i.e.*, whether the diminished but not destroyed response is due to a partial but common inactivation of all of the protein molecules, or to traces of active substance which remain uncoagulated at the final end-point of the coagulation reaction, or perhaps to a gradual *in vitro* or *in vivo* reversal of the coagulated protein to its original form.

Experiment 2. Influence of Heating at Different Temperatures upon the Antibody-Invoking Properties of Suspensions of Pneumococcus Cells.

The following experiment dealt with a comparison of the influence of the temperature of heating upon the antibody-invoking properties of suspensions of pneumococcus cells. In contrast to the solutions of bacterial substances studied in the preceding experiment, the suspensions of the bacterial cells contain two effective antigens: the antigenic complex (SP) which gives rise to the type-specific (anti-S) antibodies, as well as the species-specific antigen (P) which gives rise to the species-specific (anti-P) antibodies. Hence, this experiment consisted essentially in a comparison of the influence of the different temperatures upon two types of antigens (type-specific and species-specific) both of which are contained in the same pneumococcus cell.

The bacterial sediment of a centrifuged 12 hour broth culture was resuspended in one-tenth volume of salt solution, so that 1 cc. of the suspension was equivalent to 10 cc. of culture. The suspension was divided into four equal portions; the separate lots were then heated for 30 minutes at one of the following temperatures: 55°, 75°, 100°, and 120°C. Eight rabbits of approximately the same age and weight were selected and divided into two series. The individual rabbits in the first series received doses of vaccine equivalent to 0.5 cc. of culture and the individuals in the second series received doses equivalent to 5.0 cc. of culture; one animal in each series being injected with vaccine which had been heated at one of the four test temperatures. The immunization consisted of three courses of six daily

intravenous injections, with 1 week's rest between each course. 10 days after the last injection, the animals were bled, and the immune serum tested for type-specific and species-specific antibodies by the methods described.

The immune serum obtained with suspensions of pneumococci which had been heated at different temperatures (55° to 120°C.) contained the usual type-specific antibodies, regardless of the temperature at which the vaccine had been heated. However, the response of the individual animals to the different amounts of the same vaccine did not show the definite relation between amount of antigen and degree of antibody response which was evidenced in the previous experiment with the pneumococcus solutions. The serum from the rabbit injected with the smaller dose frequently contained as great an amount of type-specific antibody as did the serum obtained from animals which had received doses 10 times as large of the same material. Due to the irregularity of response and to the lack of a definite difference between the responses to the two test doses of vaccine heated at the same temperature, it was impossible to show from this experiment any general distinction between the influence of the different temperatures upon the effectiveness of the type-specific and species-specific antigens. The results as a whole, however, indicated that heating at any temperature had less effect upon the effectiveness of the type-specific antigen than upon the effectiveness of the species-specific antigen.

Experiment 3. Comparison of the Antibody (Type-Specific and Species-Specific) Response to Unheated Suspensions of Pneumococci with the Response to Heated Suspensions of Pneumococci.

The results of the preceding experiment had shown no definite difference between the responses to the pneumococcus suspensions which had been heated at different temperatures. It seemed probable that a more clean-cut distinction between the type-specific and species-specific antigens would be evidenced by limiting the comparison to the differences in the antigenic effectiveness of unheated and heated suspensions, rather than by attempting to show differences in the effect of heating at different temperatures.

In the following experiment, one series of rabbits was injected with unheated or live suspensions, and two other series were injected with suspensions heated at 55° and at 100°C. Since it was desired, if possible, to base our comparisons upon test doses which showed some direct relation between dosage and degree of response to the same lot of vaccine, it was decided to increase the difference between the test doses employed in Experiment 2, and to use for the larger dose in each series, an amount of vaccine 15 times as great as that represented by the smaller dose. The immunization was continued for four courses of injections with bleedings after the second and fourth courses. The use of two test doses and the examination of the immune serum after two and four courses of injections furnished a more valid basis for a distinction between the effect of heat upon the antigenic

effectiveness of the type-specific and species-specific antigens of the pneumococcus cell.

Twelve male rabbits of approximately the same age and weight were divided into three series. The first series of four animals received one course of injections of pneumococcus suspension which had been heated 8 minutes at 55°C. and three courses of unheated suspension. The second and third series of rabbits received four courses of the same suspension of pneumococci differing only in that the suspension had been heated for 30 minutes at 55° and at 100°C., respectively. Two rabbits in each series received daily doses equivalent to 0.4 cc. of broth culture, and the other two rabbits in the series received doses 15 times as large (equivalent to 6.0 cc. of culture). Each course of injections consisted of six daily doses with the usual period of 1 week between the first and second, and between the third and fourth courses. Bleedings were made 10 days after the last injection of the second and fourth courses, which caused an 11 day rest period between the second and third courses. Two rabbits (Nos. 25 and 29) died during the third course of injections.

The results of tests of the immune sera are collected in Table II.

The results in Table II can be analyzed as follows: After both the second and fourth courses of injections, the sera of all of the animals which had received the larger amount of vaccine contained a greater amount of type-specific antibody than did any of the animals which had received the smaller test dose. This is important, for the fact that the smaller test dose was less than that required for the maximum antigenic response furnishes a more desirable basis for a comparison of the effect of heat upon the effectiveness of the two types of pneumococcus antigens than if both test doses had been above that required for the maximum response.

If a comparison is made of the type-specific antibody responses to the larger test dose of vaccine, it is evident that no distinction can be made between the sera obtained by immunization with unheated pneumococcus cells and that obtained by immunization with suspensions which had been heated either at 55° or at 100°C. The same general relation holds true in the sera obtained by immunization with the smaller dose: although none of these sera contain as high a content of anti-S antibodies as do the sera obtained by use of the larger dose of vaccine, the use of the unheated bacterial cells did not cause a more effective response than did the pneumococci which had been heated. The results obtained with the animals immunized with the smaller doses are in one respect more convincing than that obtained

TABLE II.

Comparison of Antibody-Involving Property of Unheated Suspensions of Pneumococcus Cells with That of Suspensions Which Had Been Heated at Different Temperatures.

Antigen used in immunization Suspension of pneumococcus cells			Species-specific anti-protein precipitins		Type specific antibodies							
			Serum after 2 courses of injections	Serum after 4 courses of injections	Serum after 2 courses of injections				Serum after 4 courses of injections			
					Type II agglutinins		Passive protection		Type II agglutinins		Passive protection	
					Dilution of serum		Dose of culture		Dilution of serum		Dose of culture	
Rabbit No	Treatment of antigen	Dose in terms of broth culture	Precipitation with $\frac{1}{2}$ dilution of test solution	Precipitation with $\frac{1}{4}$ dilution of test solution	1/10	1/80	0.1 cc	0.01 cc	1/10	1/80	0.1 cc	0.01 cc
18	Unheated	6.0	+	+++	xxx	r	S	S	xxx	x	S	S
19	Unheated	6.0	+	+++	xxx	x	S	S	xxx	r	S	S
20	Heated 30 min at 55°C.	6.0	±	+	xxx	r	S	S	xxx	r	S	S
21	Heated 30 min at 55°C	6.0	±	+	xxx	0	S	S	xxx	x	S	S
22	Heated 30 min at 100°C	6.0	±	+	xxx	r	S	S	xxx	x	S	S
23	Heated 30 min at 100°C	6.0	=	+	xxx	0	S	S	xxx	r	S	S
24	Unheated	0.4	±	+++	r	0	D	S	xx	0	S	S
25	Unheated	0.4	±	*	xx	0	S	S	*	*	*	*
26	Heated 30 min at 55°C	0.4	0	0	xx	0	S	S	xx	0	S	S
27	Heated 30 min. at 55°C	0.4	0	0	x	0	D	S	r	0	D	S
28	Heated 30 min at 100 C.	0.4	0	0	x	0	D	S	xx	0	S	S
29	Heated 30 min. at 100°C.	0.4	0	*	r	0	D	S	*	*	*	*

* Rabbit died during immunization

In Tables II and III 0 = no detectable reaction; ± = faint cloudiness without sedimentation; + = cloudiness with slight sedimentation, ++ = marked cloudiness with moderate sedimentation; +++ = marked cloudiness with large amounts of sediment, x = definite granulation without formation of compact disc, xx = compact disc with faintly cloudy supernatant fluid, xxx = compact disc with clear supernatant fluid; D = mouse died in 18-60 hours; S = mouse survived 7 days.

with the larger test dose, for the fact that the test dose is known to be less than that required for the maximum response indicates that the lack of any essential difference in apparent antigenic effectiveness is not obscured by the injection of excessive amounts of total antigen.

Quite different relations are revealed in a comparison of the species-specific antibody (anti-P) response. In the case of the animals injected with the larger test dose, the sera obtained by immunization with the unheated pneumococci contain much more of the species-specific antibody than do those immunized with the heated bacterial cells. While the sera of the rabbits receiving the heated material increased in their anti-P precipitin content as the immunization was continued, it is evident that even after four courses of injections none of these animals produced more of the species-specific antibody than that invoked in other animals by two courses of injections of the unheated material. The same distinction is revealed in the results obtained in the animals which received the smaller test dose. Again, the animals which received unheated pneumococci produced more anti-P than did those which received the same amount of heated bacteria. The distinction between the anti-P response to the heated and the unheated bacterial cells is in striking contrast to the apparent lack of any difference in the anti-S response to equal doses of heated and unheated pneumococcus suspensions. Whether or not the bacterial cells have been heated is apparently the most important factor in determining the relative amount of the species-specific antibody in antipneumococcus sera, for although size of dose and length of the period of immunization also increased the anti-P response to the heated bacterial suspension, doses 15 times as great of the heated bacteria failed to invoke as effective an anti-P response as that obtained by small doses of unheated pneumococci.

These results are of additional interest as further evidence of the individuality of the type-specific and of the species-specific antigens of *Pneumococcus*, for the existence of two different antigens which are affected to a different extent by heat are required in order to obtain immune sera in which the relative proportion of the two antibodies is determined by the use of unheated or heated bacterial cells in the immunization.

Experiment 4. Comparison of the Antibody Response to Subsequent Injections of Unheated and of Heated Pneumococcus Cells by Rabbits Previously Immunized with Heated Pneumococci.

The following experiment differs from the preceding ones in that the animals received a preliminary immunization treatment with heated pneumococcus cells; and were then divided into two groups, one of which was given additional immunization with heated pneumococci, and the second was given additional immunization with unheated pneumococci.

It is a common practice in the preparation of antibacterial serum to employ heated bacteria in the first part of the immunization treatment and to inject live bacteria in the later stages of the immunization in the hope of increasing the protective value of the serum. Since passive protection of mice against Type II pneumococci is apparently a function of the type-specific antibody (5), the results of the preceding experiments furnished considerable ground for the belief that unheated pneumococci would be no more effective than heated bacterial cells in the production of a serum of high protective or high agglutinating value. However, in view of the possibility of differences in the responses of previously immunized animals, the following experiment was designed to compare the responses induced by subsequent immunization with unheated bacteria with the responses induced when the immunization was continued with heated bacteria alone. All of the animals selected for the final test immunization, had responded to previous immunization with the heated bacteria with the production of immune serum of high titres of type-specific antibody before the final test comparison of the effect of heat upon the bacterial cells injected in the subsequent immunization.

Eighteen rabbits were immunized with three courses of daily injections of heated vaccine, each dose being equivalent to 5.0 cc. of broth culture. The immune sera were then tested by the usual methods for type-specific and species-specific antibodies. From the original eighteen animals, twelve were selected for the final test comparison. Six rabbits which gave weak responses to the preliminary immunization were eliminated from the experiment. Each of the selected twelve rabbits had produced, in response to the preliminary immunization, sera which contained high and approximately equivalent amounts of the usual type-specific antibody (anti S precipitins, agglutinins, and passive protective action). Since the preliminary immunization consisted of eighteen injections of relatively large amounts of vaccine, it was assumed that at this stage the animals had had sufficient immunological stimulus to invoke an anti-S response approximately equivalent to the responding capacity of the individual animals. It is obvious, thus, that the following comparison of the effect of subsequent immunization with unheated and with heated bacterial cells, was limited to animals which had already given evidence of effective response to heated pneumococci and which had in fact already produced as high titres of type-specific antibody as is usually obtained in

Type II antipneumococcus rabbit serum. None of the animals had produced more than the small amount of the species-specific antibody commonly found in antipneumococcus serum of rabbits immunized with heated vaccine.

These twelve previously immunized rabbits were then divided into two series: Series *A* received heated pneumococci in the subsequent immunization; Series *B* received the same amount of unheated pneumococci; the subsequent immunization consisted of two courses of six daily injections of bacterial suspension equiva-

TABLE III.

Comparison of the Antibody Responses to Subsequent Injections of Unheated and Heated Pneumococci by Rabbits Previously Immunized with Heated Pneumococci.

Rabbit No.	Treatment of pneumococcus suspension injected in subsequent immunization	Type-specific antibody									Species-specific antibody				
		Agglutinins				Passive protection					Antiprotein precipitins				
		After 3 preliminary courses of heated pneumococcus		After subsequent immunization		After 3 preliminary courses of heated pneumococci			After subsequent immunization		After 3 preliminary courses of heated pneumococci		After subsequent immunization		
		Serum dilution				Dose of culture						Dilution of test solution			
		1/20	1/80	1/20	1/80	0.2 cc.	0.1 cc.	0.03 cc.	0.2 cc.	0.1 cc.	0.03 cc.	1/4	1/20	1/4	1/20
30	Heated	xx	x	xx	0	D	D	S	D	S	S	+	±	+	±
31	"	xx	0	xxx	0	D	S	S	S	S	S	±	0	+	±
32	"	x	0	xx	0	S	D	D	D	S	S	0	0	±	0
33	"	xx	x	xxx	x	D	S	S	D	D	S	±	0	±	0
34	"	xx	0	xxx	x	S	S	S	S	S	S	±	0	+	±
35	"	xxx	0	xxx	0	S	S	S	S	S	S	±	0	±	0
36	Unheated	x	0	xx	0	S	D	S	S	S	S	+	±	++	+
37	"	xx	0	xx	0	S	D	S	D	S	S	0	0	++	+
38	"	xx	0	xxx	x	D	S	S	S	S	S	0	0	++	+
39	"	x	0	xx	0	D	D	S	D	S	S	±	0	++	+
40	"	xx	0	xxx	x	D	D	S	S	D	S	±	0	++	+
41	"	xx	0	xxx	0	D	D	S	D	S	S	±	0	++	+

lent to 5.0 cc. of broth culture. After the end of this treatment, a second bleeding was made, and the immune serum obtained was tested by the same methods employed in the tests of the first bleeding.

The results are collected in Table III. Since the anti-S precipitins and the type-specific agglutinins proved approximately parallel in all the sera, the results of the anti-S precipitin tests are omitted in Table III.

The facts presented in Table III can be analyzed to advantage by considering first the type-specific antibody response. All of the animals in both Series *A* (heated) and *B* (unheated) had produced sera containing high titres of the type-specific antibody after the first three courses of injections. This response apparently represented the maximum response of the individual animals, since only slight increases and at times slight decreases in the content of type-specific antibody was obtained by continued immunization with either heated or with unheated pneumococci. Apparently, with the selected previously immunized rabbits used in this experiment, the slight differences that existed in the potency of the sera of the different animals was determined by the capacity of the animal to respond and was independent entirely of whether or not the bacterial cells had been heated.

This apparent total lack of any real difference between the type-specific antibody content of the sera obtained by subsequent injection of the previously immunized animals with unheated pneumococci in comparison to that obtained with heated bacterial cells was just as pronounced in passive protection experiments as in agglutination tests. All of the sera protected against about 0.1 cc. of culture and attempts to show differences by increasing the test doses failed due to the irregularities usually encountered in protection tests with doses of culture near the zone of mass infection. Other experiments were made in which a constant dose of culture (1×10^{-4} cc.) was tested against different amounts of serum. The results of these experiments showed some differences in the potency of the different sera but the differences were irregular and had no relation to the heating treatment of the immunization material.

An analysis of the species-specific antibody responses induced in the previously immunized rabbits reveals entirely different relations than those obtaining for the species-specific antibody. The sera of all the rabbits contained only small amounts of the anti-P precipitin at the end of the preliminary immunization which, as shown before, had yielded sera containing high contents of anti-S. When these previously immunized animals were subsequently injected with additional doses of heated pneumococci there was only a slight increase in the species-specific antibody yield. On the other hand, the animals which

received unheated pneumococci in the subsequent immunization treatment responded with large amounts of the species-specific antibody so that their sera after the final two test courses of injections contained anti-P precipitins in titres comparable to those obtained by immunization with large amounts of solutions of pneumococcus cells or of pneumococcus protein.

The most important facts shown by this experiment can be illustrated best by summarizing the results of Table III in the form given

TABLE IV.

Summary of the Antibody Responses to Subsequent Injections of Unheated and Heated Pneumococci by Rabbits Previously Immunized with Heated Pneumococci.

Immunization		Increase in antibody content of sera due to subsequent immunization of the previously immunized rabbits	
		Type-specific antibodies	Species-specific antibodies
Animals previously immunized with three courses of injections of heated pneumococcus cells	Animals which subsequently received two additional courses of heated pneumococci	No significant increase	No increase by 3 rabbits; slight increase by 3 rabbits
	Animals which subsequently received two additional courses of unheated pneumococci	No significant increase	Marked increase by all (6) rabbits

in Table IV. The relations evident there (Table IV) indicate that individual rabbits can produce only a certain amount of the type-specific antibody and that the maximum response can be obtained by the injection of heated pneumococcus cells; and that after this maximum responding capacity has been reached, the further injection of unheated pneumococci serves only to increase the species-specific antiprotein precipitin content without increasing significantly the type-specific antibody content of the serum.

Experiment 5. The Antihemotoxin Response of Rabbits Immunized with Heated and with Unheated Pneumococcus Cells.

Pneumococcus antihemotoxin, like the anti-P precipitin, is a species-specific and not type-specific antibody (11). The hemotoxin, the antigen which gives rise to the antihemotoxin antibody, is an endocellular substance and like the antigens which give rise to the anti-P precipitins is present in solutions of pneumococcus cells. In a previous paper (10), it was shown that the hemotoxin can be distinguished from the antigens related to the anti-P precipitins by the fact that the antibody-invoking property of the hemotoxin antigen is destroyed by heating treatment (55°C.) which diminishes but does not destroy the effectiveness of the precipitinogens. Since the hemotoxin like the P precipitinogens is contained as an endocellular constituent in the pneumococcus cell suspensions injected for the production of the usual antibacterial antipneumococcus serum, one would expect the relative proportion of the antihemotoxin contained in antipneumococcus serum to be influenced by the heating treatment of the bacterial suspensions employed in the immunization. In fact, from analogy with the results obtained in immunization with heated solutions of pneumococcus cells, one would expect the antihemotoxin content to be almost entirely dependent upon whether the bacterial cells were heated or unheated, for we failed entirely to detect any antihemotoxin in the immune serum of animals injected with heated hemotoxin solutions (10).

In order to determine the effect of the heating treatment of suspensions of pneumococcus cells upon the antihemotoxin content of antipneumococcus serum, antihemotoxin titrations were made with the sera of the forty-one animals employed in the preceding experiments. All of the sera obtained by immunization with unheated pneumococcus cells, like the sera obtained by immunization with unheated solutions of the bacterial substances, contained significant amounts of antihemotoxin. With only one exception, none of the sera obtained by immunization with heated pneumococcus cells contained any detectable amount of the antihemotoxin. The one exception consisted in the serum of a rabbit which had received prolonged immunization with large amounts of bacterial cells heated at 100°C. (Rabbit 23 in Table II). It is impossible to explain this apparent discrepancy.

In spite of the observed exception, it is logical to believe that the antihemotoxin content of antipneumococcus serum, like the anti-P precipitin content of the same sera, is determined largely by the heating treatment of the bacterial cells employed in the immunization. One would expect to find especially large amounts of this species-specific antibody in the antipneumococcus serum obtained from horses which had received large doses of live or unheated pneumococci during the later stages of the immunization.

DISCUSSION.

The preceding experiments have dealt with the influence of heat upon the antibody-invoking effectiveness of the type-specific and species-specific antigens of Type II pneumococcus cells. The results of the study of the effect of heat upon the species-specific antigen as contained in filtered solutions of the intracellular substances of pneumococci showed that 30 minutes exposure of the bacterial solutions to 55°C. or higher temperatures, caused a marked decrease in the antibody-invoking effectiveness of the species-specific antigen, not only from the standpoint of the degree of antibody response to equal doses of unheated or of heated solution, but also from the standpoint of the amount of total antigen and number of injections required for an effective response. The experiments with suspensions of pneumococcal cells were more complicated than those in which solutions were used as the immunization material, since the latter experiments included the type-specific as well as the species-specific antibodies. More differences existed in the responses of individual animals immunized with the same lot of bacterial suspension than were encountered among the rabbits immunized with the bacterial solutions. However, the large number of rabbits included, the use of different doses in the immunization, and the examination of the immune serum after different periods of immunization, furnished convincing evidence that the antibody-invoking property of the species-specific antigen as contained in the suspensions of bacterial cells was more greatly diminished by heat than was that of the type-specific antigen. For example, a series of rabbits injected with large but equal doses of heated (30 minutes at 55° or 100°C.) or of unheated suspensions of pneumococci, contained in common a high content of type-specific antibody independent of the heating treatment of the antigen; and in contrast to their common content of the type-specific antibody, the same sera contained large amounts of the species-specific antibody only where unheated bacteria had been employed in the immunization. Moreover, a comparison of the sera of another series of rabbits showed that the immune serum obtained by injection of small amounts of unheated pneumococcus suspension (below the dose required for the most effective response) contained less type-specific but more species-

specific antibody than did the serum of other rabbits which had received doses 15 times as great of the heated bacterial cells. The production of immune serum in which the relative proportion of type-specific to species-specific antibody seems to be directly dependent upon whether or not the immunization material had been heated or unheated, presents convincing evidence of differences in the effect of heat upon the antibody-invoking effectiveness of the type-specific and species-specific antigens of *Pneumococcus*. It is important to observe that these relations were established in experiments in which dosage and length of time of immunization were carefully controlled, for it would be theoretically possible to obtain similar responses to heated and unheated antigen if the total amount of antigen were greatly in excess of the dose required for the maximum immunity response.

The species-specific antigen of *Pneumococcus* is endocellular and it can be assumed that the bacterial cells must be dissolved, either before or after injection into the animals, in order to liberate the antigen and permit it to come into direct contact with the cells involved in antibody production. Since unheated pneumococci autolyze *in vitro* more readily than do the heated bacterial cells and are likewise more soluble in bile, the question might be raised whether a greater rate of lysis of the unheated cells *in vivo* may not be a factor in the greater effectiveness of the endocellular antigen. But *in vivo* and *in vitro* dissolution of pneumococci are distinctly different processes: one of them due in large part to the specific heat-labile bacteriolytic enzyme produced by the bacteria themselves (12) and the other to lytic action of the cells or fluids of the animal. However, the possibility of differences in the ease of lysis of the unheated and heated pneumococci, while not to be ignored, does not seem to be an important point for the fact that the antibody-invoking effectiveness of the protein as contained in the solutions employed in the first experiment was influenced as markedly by heat as was the protein contained within the undissolved cells in later experiments, indicated that the decrease in antigenic activity represents a real change in the bacterial protein which occurs whether heated inside or outside the bacterial cell.

The distinct difference in the relative effect of heat upon these two antigenic constituents of the pneumococcus cell is of interest from both theoretical and practical points of view. It is important to note that

our experiments have dealt with the antibody-invoking and not with the antibody-combining property of the two antigens. The type-specific S substance when free in solution, dissociated from the antigenic complex SP, does not possess the antibody-invoking property whether heated or unheated, but it retains its antibody-combining property after even the most drastic heating treatment (in approximately neutral solutions). The resistance of the antibody-combining property of the S substance to the autoclaving and boiling in the open water bath, both of which are preliminary steps in the preparation of the purified substance (1, 2), is an outstanding example of heat stability among immunologically reactive substances. However, even the antigenic complex itself (SP), undissociated and in the antibody-invoking effective form, is relatively heat-resistant in comparison to many antigens of bacterial origin and, from the antibody-invoking standpoint is decidedly more heat-stable than the species-specific protein antigens contained within the same pneumococcus cell. Possibly, combination with the carbohydrate or S substance endows pneumococcus protein with new physical properties (resistance to heat coagulation) as well as conferring upon it the new chemical or immunological properties which are responsible for type specificity.

The difference in the heat lability of the type-specific and species-specific antigens of *Pneumococcus* must be an important factor in determining the relative proportion of the type-specific and species-specific antibodies in all antipneumococcus serum produced by injection of pneumococcus suspensions. While the type-specific antibody (anti-S) is the predominant and important one, a certain amount of the species-specific antibody accompanies it in practically all type-specific sera. The species-specific antibody content and the ratio between type-specific and species-specific antibody vary in sera produced in different laboratories; one serum may contain relatively large amounts of the species-specific antibody and another may contain only difficultly detectable traces of the same antibody, even though the type-specific antibody content of the two sera is approximately identical. The factors which operate in determining the relative amount of species-specific antibody must include those which determine the degree of the response to any antigen: treatment of the immunization material, dosage, period of immunization, and the re-

sponding capacity of the individual animal. However, if the vaccine is prepared from virulent, S-producing pneumococci and reasonable doses are injected in a course of immunization not unnecessarily long, one could expect that heating of the pneumococcus suspension injected would greatly decrease the production of species-specific antibodies (anti-P) without an appreciable effect upon the production of type-specific antibodies (anti-S). This is shown to be true with rabbits in the preceding experiments.

The results of the experiments on subsequent immunization of rabbits previously immunized with heated pneumococci, indicated that the only thing gained by the subsequent immunization is an increase in the species-specific antibody content of the serum. Since this latter antibody is not involved in truly type-specific agglutination, the conclusion may be drawn that continued immunization of animals which already have responded with high titres of type-specific antibody adds nothing at all to the diagnostic value of the serum. The use of live or unheated bacteria is unnecessary in order to produce a highly potent type-specific sera, and, if the sera are to be used for typing, the increase in species-specific antibody without increase in the type-specifically reacting substances is undesirable.

SUMMARY.

This paper presents an experimental comparison of the effect of heating of the immunization material upon the antibody-invoking effectiveness of the type-specific (SP) and species-specific (P) antigens of Type II pneumococci. Heating of the pneumococcus suspension (vaccine) invariably decreased the production of species-specific antibodies (anti-P) without a comparable effect upon the production of type-specific antibodies (anti-S).

For diagnostic typing purposes, the ideal antipneumococcus serum should contain the maximum content of type-specific, and the minimum of species-specific antibody. Our results with forty-one rabbits indicate that the ideal serum from the type-specific standpoint would be obtained by immunization with the heated cells of virulent pneumococci over a comparatively short immunization period; and that the only thing gained by continued immunization or by the use of unheated bacteria at any stage of the immunization, is an increase in

the species-specific antibody which is undesirable in sera to be used for diagnostic purposes.

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STUDIES ON STREPTOCOCCUS BACTERIOPHAGE.

II. THE INFLUENCE OF LYTIC PRINCIPLES UPON THE AGGLUTINATION OF HEMOLYTIC STREPTOCOCCI.

BY GREGORY SHWARTZMAN, M.D.

(From the Laboratories of the Mount Sinai Hospital, New York.)

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INTRODUCTION.

Bacteriophage is capable of inducing considerable disturbance in susceptible bacterial cells. Especially interesting is the influence of this principle upon the agglutinative properties of bacteria. The studies on the subject reported by d'Hérelle (1), Bordet and Ciuca (2), Gratia (3), Flu (4), McKinley (5) and others were limited to the *coli*-typhoid-dysentery group of bacteria. The observed changes consisted of loss of agglutinability by specific sera with and without loss of agglutinogenic and agglutinin-absorbing properties. In one instance, as reported by Gratia (3), the effect of the lytic principle consisted in appearance of agglutinability in a previously inagglutinable strain of *B. coli*.

The author's object was to extend these studies to other bacteria in which the bacteriophage phenomenon was shown to exist. The hemolytic pathogenic streptococci were chosen for this work for the following reasons:

1. It was shown by Dutton (6) that many strains of human pathogenic streptococci existed symbiotically with phage of low potency. Two strains of human origin were shown to undergo lysis (Piorkowski (7) and Dutton (6)). The author demonstrated the classical bacteriophage phenomenon among several strains of erysipelas origin (8). Since, therefore, it can be safely accepted that the bacteriophage phenomenon exists among streptococci, it seemed possible that there might exist a definite influence of phage upon the phenomenon of agglutination of these microorganisms.

2. The complexity of the problems of serological specificity and affiliations of different groups of streptococci makes it important to determine the influence of the phage which represents a powerful factor in the life of many species of bacteria, including streptococci.

The plan of this work was influenced by the following considerations:

Since the effect of the phage upon agglutination of streptococci is entirely unknown it was decided to study the effect of the phage upon the entire strain. Later should a definite relation be established, these studies could be extended to strain components derived from single colonies or single cells.

In view of the fact that various strains of pathogenic streptococci differ in the degree of susceptibility to the phage it was presumed that the changes induced would vary as well. For this reason a large number of strains was employed.

Since various groups of hemolytic streptococci differ in the complexity of the antigen it was also thought that the same agent would induce different changes, the nature of which might depend upon the antigenic structure. This consideration led to the study of representatives of various serological groups of streptococci.

Moreover, since lytic principles from various sources differ in their influence upon streptococci (Shwartzman (8)) it was decided to limit the work to the study of one or two lytic principles and employ these throughout the work.

In order to be able to obtain a full conception of the various possible changes which this principle may induce in streptococci under the conditions outlined above, the studies were made first by means of direct *agglutinations*. The information gained was further substantiated by studies on the *agglutinin-absorbing and agglutinogenic properties* of each culture under discussion. To study the effect of phage upon the affiliations between various streptococci each serum was tested against the majority of strains on hand.

Methods.

Lytic Principles.—SL bacteriophage, active against a streptococcus from rabbit's lung and also potentially active against streptococci of erysipelas origin; the C/2, anti-*B. coli* phage were used in these experiments. Cultures of various streptococci were grown in phosphate broth containing 1 to 10 dilution of lytic

principle for a number of generations. These cultures will be referred to as SL and C/2 respectively. The strains employed in this work were as follows:

1. The erysipelas group, represented by ten strains kindly sent to me by Dr. Konrad Birkhaug under the name E₁-E₁₀. All these strains were potentially susceptible to SL lytic principle only.
2. Scarlet fever strains (55, 108), sent to me by Dr. A. W. Williams, which were found resistant to both lytic principles.
3. A strain of *pyogenes* hemolytic streptococcus, isolated in this laboratory from a case of meningitis and called H.S. This was resistant to both principles.
4. A strain of green-producing streptococcus, isolated from the blood of a case of subacute bacterial endocarditis, under the name V. This was also resistant to the lytic principles employed.
5. Rb streptococcus, a pyogenic hemolytic strain isolated by Clark and Clark from rabbit lung infection. This streptococcus was highly susceptible to SL bacteriophage and resistant to C/2 lytic principle.

Immunization.—Two rabbits were immunized with each strain and the serum of higher titer was selected for the work. The method of immunization was that recommended by Dochez (9).

Agglutination.—Homogenous antigens were obtained by growing streptococci in 0.1 per cent glucose phosphate broth. Phage strains were passed through phosphate broth containing 1 to 10 dilution of lytic principle. Both normal and phage cultures were subcultured into phosphate broth and used for agglutination after 24 hours incubation. A considerable number of experiments was first performed with the washed sediment of these cultures. The process of centrifuging, however, brought about frequent spontaneous agglutination of previously homogenous antigens. This procedure was then given up. Care was always taken to use cultures not older than 24 hours. Parallel experiments did not show any difference in agglutination of washed and unwashed 24 hour old cultures. No formaldehyde was added to the antigens. Serum dilutions were made in phosphate broth. Readings were made after 4 hours incubation in a water bath at 55°. The agglutinations registered in the protocols were examined by the naked eye.

Absorption of Agglutinins.—Bacterial cells for absorptions of agglutinins were prepared by centrifuging and washing the growth of 24 hour old broth cultures. A dose sufficient to absorb completely agglutinins from the homologous serum was used. In making comparative studies on the agglutinin-absorbing power of various antigens the quantitative relationship of absorbing cells to the amount of serum (usually diluted 1 to 10) was kept in mind. The mixture of given amounts of cells (heated previously to 60°) and serum was kept in the water bath at 37° for 2 hours and in the refrigerator overnight. In order to titrate the absorbed sera, dilutions from 1 to 20 up to 1 to 25,000 were always made. Serum diluted 1 to 10 but unabsorbed, to be used for control titration, was kept under the same temperature conditions as the serum for absorption.

The results reported below were grouped according to the changes produced by given lytic principles in the various strains of pathogenic streptococci.

EXPERIMENTAL.

I. Inagglutinability and Partial Loss of Agglutinogenic and Agglutinin-Absorbing Properties of "Whole" Cultures.¹

Strain 55 which represented serologically a large group of scarlet fever streptococci was cultured for forty passages in phosphate broth and in phosphate broth containing SL phage. The effect of the phage is shown in Table I.

TABLE I.
The Influence of Phage upon Agglutination of Strain 55.

Sera	Absorbed by strain	Agglutination titer before absorption and percentage of absorption	
		55	55 SL
I	=	6400	0
I	55	100 per cent	—
I	55 SL	50 per cent	—
II	=	400	0
III	=	800	0

0, no agglutination; = no absorption; — not tested.

I, 55 strain serum; II, 55 SL strain serum; III, 55 SL strain serum.

As is seen the phage strain was rendered completely inagglutinable by normal culture serum. This change was coincident with partial loss of agglutinin-absorbing properties and also with what appeared a certain loss in the agglutinogenic power. In fact the immunization of two animals with the phage cultures resulted in a distinctly lower titer of agglutinins. These sera agglutinated the normal culture but did not react with the homologous culture.

Similar but somewhat more marked changes were obtained in Rb streptococcus.

¹ The change demonstrated in this work occurred in entire cultures. It remains yet to determine whether this was due to actual changes in the cells bearing the agglutinogens or to simple elimination of these cells by means of the lytic agent.

This streptococcus highly susceptible to SL phage was made resistant to this principle by means of several passages through broth containing at first 1×10^{-6} cc. and later 1×10^{-1} cc. dilution of the phage. Observations on cross-agglutinations and cross-absorptions of sera prepared by immunization with normal Rb streptococcus, the resistant type and lysed culture of the same strain are given in Table II. As is seen, Rb streptococcus was considerably affected by passage through SL phage. The strain became inagglutinable by the normal culture serum as well as by heterologous sera which normally cross-

TABLE II.

The Influence of Phage upon Agglutination of Rb Streptococcus.

Sera	Absorbed by strain	Agglutination titer before absorption and percentage of absorption			
		Rb streptococcus	Resistant Rb streptococcus	55	130
IV	=	6400	0	400	40
IV	Rb streptococcus	100 per cent	—	0 per cent	0 per cent
IV	Resistant Rb. streptococcus	0 per cent	—	—	—
V	=	50	0	0	0
VI	=	0	0	0	0
VII	=	0	0	0	0
VIII	=	0	0	0	0
IX	=	0	0	0	0
Ia	=	200	0	5120	400
X	=	200	0	—	3200

IV, Rb strain serum; V, VI, VII, lysed culture Rb strain serum; VIII, IX, resistant Rb strain sera; X, 130 strain serum (55 and 130 belong to the scarlet fever group); Ia, 55 strain serum.

agglutinated with this strain. The inagglutinability was coincident with considerably more marked changes of the antigen than those shown in the previous experiments. There was a very significant loss of agglutinin-absorbing power on the part of the resistant type and also what appeared as an entire loss of agglutinogenic properties in both lysed and resistant cultures. Five animals failed to respond to repeated immunization with these antigens. Additional attempts to immunize with such a strain are necessary before complete loss of the agglutinogenic properties can be safely accepted.

It should be pointed out that the group components of this strain underwent similar changes. In contrast to the normal Rb culture the resistant streptococcus failed to agglutinate with Strains 55 and 130 and also failed to stimulate the production of group agglutinins for the two strains.

II. Inagglutinability with Complete Preservation of Agglutinogenic and Agglutinin-Absorbing Properties. Appearance of Additional Components.

Examples of partial modification of antigens under the influence of phage were afforded by representatives of various groups of streptococci:

A. The Erysipelas Group of Streptococci.—Table III represents the results of direct agglutinations of normal and phage cultures of erysipelas strains. As is shown by Birkhaug (10), Dochez (9) and Tunnicliff (11) this group of streptococci possesses a specific antigen. In this work the serum for one of these strains agglutinated to a high titer several representatives of this group. The bacteriophage had a definite effect upon these strains. SL lytic principle, potentially active against these strains, lowered the agglutinability of eight strains out of ten tested. This change, however, did not alter the specific component of the strains since the agglutinin-absorbing power of all the phage cultures remained unaffected. The specific agglutinins of Serum XI were completely absorbed by these cultures (not recorded in the tables). Moreover, Serum XII agglutinated the normal strains to a high titer, as is seen from Table III. However, another component was rendered prominent in the phage cultures, as evidenced by the following observation. The phage culture serum (XII) agglutinated all the phage cultures to a higher titer than the normal cultures when tested soon after bleeding (not given in Table III). Since these strains agglutinated to a lower titer with the normal culture serum it suggested that another component was rendered prominent in the phage cultures.

This assumption was further strengthened by another observation. As is seen from Table III, when sterile, non-preserved Serum XII was retested 3 weeks after bleeding, the phage cultures this time agglutinated to a lower titer than the normal cultures. A new phage culture serum (XIII) was then prepared and a part of it was added with

TABLE III.
The Influence of Phage upon Agglutination of Erysipelas Streptococci.
Agglutination Titer.

[illegible]

XI, E₁ normal culture serum; XII, E₁ SL phage culture serum; XIII, E₁ SL phage culture serum.

phenol.² The new serum, sterile but not preserved, when tested the following day after bleeding showed agglutination of a higher titer with phage cultures. Part of Serum XII, to which phenol was added immediately after bleeding when tested 3 weeks after bleeding also showed higher agglutination with the phage cultures. Serum XIII, preserved with phenol, titrated 2 weeks after bleeding, showed the same titer as the same serum non-preserved but tested immediately after bleeding. It became thus evident that the phage culture stimulated production of agglutinins specific to all the phage cultures. These agglutinins were of unstable nature and unless the serum was preserved with phenol, they soon disappeared. The relation of this additional component to the specific erysipelas component was established by cross-absorption. Four normal cultures absorbed completely all the agglutinins from phenolized Sera XII and XIII. Each of four phage cultures employed absorbed agglutinins for all the phage cultures. It was clear that the additional phage culture component was closely related to the specific erysipelas streptococcus antigen and that the phage culture components of various erysipelas streptococcus strains were closely affiliated as well.

B. The Scarlet Fever Group of Streptococci.—Strain 108 was cultivated for twenty-two passages in phosphate broth and in phosphate broth containing SL phage. The phage culture was studied as shown in Table IV. As is seen, Strain 108 became under the influence of phage relatively inagglutinable by the normal culture serum (XIV). This change, however, was not coincident with loss of agglutinin-absorbing and agglutinogenic properties, since, 108 phage culture completely absorbed Serum XIV and also stimulated production of agglutinins for 108 normal culture. Parallel to the inagglutinability of the specific 108 component another antigen appeared in the phage culture. This was evident from the fact that Serum XV, in contrast to Serum XIV, agglutinated the phage culture as well as the normal culture of Strain 108. Since, however, the normal 108 culture was able to absorb completely agglutinins for the phage culture from Serum

² All the sera including Serum XII were collected under sterile precautions by heart puncture and not preserved by phenol. Then the animals were killed, the blood clot removed and its serum preserved with phenol.

XV it was concluded that the phage culture component was closely related to the 108 normal culture specific antigen.

To complete the investigation of the phage culture component, its relation to another strain of the scarlet fever group was studied by cross-agglutinations and cross-absorptions. Strain 55, chosen for this purpose, had common components with Strain 108 in addition to a heterologous antigen (Table IV). It was then observed that 108 phage culture serum agglutinated Strain 55 to a far higher titer than that shown by 108 normal culture serum. Normal culture 55 serum

TABLE IV.

The Influence of SL Phage on Agglutination of 108 Strain.

Sera	Absorbed by strain	Agglutination titer before absorption and percentage of absorption		
		55	108	108 SL
XIV	=	400	3200	200
XIV	55	100 per cent	0 per cent	0 per cent
XIV	108	100 per cent	100 per cent	100 per cent
XIV	108 SL	100 per cent	100 per cent	100 per cent
XV	=	1600	1600	3200
XV	55	100 per cent	0 per cent	0 per cent
XV	108	100 per cent	100 per cent	100 per cent
XV	108 SL	100 per cent	100 per cent	100 per cent
I	=	6400	400	3200
I	55	100 per cent	100 per cent	100 per cent
I	108	50 per cent	100 per cent	100 per cent
I	108 SL	25 per cent	100 per cent	100 per cent

XIV, 108 normal culture serum; XV, 108 SL phage culture serum.

agglutinated the 108 phage culture to a considerably higher titer than the normal culture of the same strain. It became, therefore, apparent that the phage culture 108 components belonged to the "group" variety. However, Strain 55 absorbed agglutinins for itself but failed to absorb agglutinins for 108 phage culture from Serum XV. It had to be assumed that the phage components were of a more complex structure than the 55 strain group antigen.

The conclusion to be drawn was that the phage was able to render prominent additional components of a complex antigenic structure. These components were partially of the "group" variety and, there-

fore, made possible a certain measure of agglutination with a heterologous strain.

C. Rb Streptococcus.—Another example of partial modification was brought out by Rb streptococcus which was cultivated in C/2 phage. It should be pointed out that the strain was completely resistant to this phage. Table V represents the results obtained. As is seen, here again Rb streptococcus C/2 culture became inagglutinable by the normal culture serum. The inagglutinability was not coincident with any changes in agglutinin-absorbing and agglutinogenic proper-

TABLE V.

The Effect of C/2 Phage upon Agglutination of Rb Streptococcus.

Sera	Absorbed by strain	Agglutination titer before absorption and percentage of absorption		
		Rb streptococcus	Rb C/2 strain	V
XVI	=	6400	0	0
XVI	Rb streptococcus	100 per cent	—	—
XVI	Rb C/2	85 per cent	—	—
XVI	V	0 per cent	—	—
XVII	=	0	1600	3200
XVII	Rb streptococcus	—	0 per cent	0 per cent
XVII	Rb C/2	—	100 per cent	100 per cent
XVII	V	—	100 per cent	100 per cent
XVIII	=	1600	800	800
XVIII	Rb streptococcus	100 per cent	0 per cent	0 per cent
XVIII	Rb C/2	100 per cent	100 per cent	100 per cent
XVIII	V	0 per cent	100 per cent	100 per cent

XVI, Rb streptococcus serum; XVII, V strain serum; XVIII, Rb C/2 strain serum.

ties since Serum XVIII showed agglutinins for Rb streptococcus and the Rb C/2 strain absorbed agglutinins from Serum XVI for the normal culture of this streptococcus. The change was, however, accompanied by the appearance of another component in the Rb C/2 strain, since its serum, in contrast to the normal Rb streptococcus serum, agglutinated the homologous as well as the normal culture to a high titer. The additional component of the phage culture was not related to the specific Rb streptococcus antigen, as judged from the inability of the Rb streptococcus to absorb agglutinins for Rb C/2 strain from Serum XVIII.

To investigate the nature of the additional phage components Serum XVIII was tested against several strains of streptococci. This serum was able to cross-agglutinate with a strain of green-producing streptococcus. No cross-agglutination was obtained with any hemolytic streptococci tested (the negative findings are not given in Table V). The green-producing streptococcus serum was then found to agglutinate the Rb C/2 strain. Moreover, cross-absorption experiments established the very close similarity of the V specific component with the additional phage culture components. In this case the added component was of the "group" variety. In the case of scarlet fever streptococci a similarly arising component was found related only to the "group" component of another strain of scarlet fever streptococci. In the present example, however, the phage component was related very closely to the specific antigen of a strain of heterologous streptococci.

III. Transformation of the Normal Culture Agglutinogens into Antigens of an Entirely Different Specificity.

As is seen from Tables VI and VII, H.S. and V streptococci when treated with SL phage for a number of generations underwent an even more striking modification. The changes consisted of complete disappearance of normal antigen and appearance of a different antigen, which had the power of absorption, agglutinability and agglutinin-stimulating properties characteristic of a new specificity. The changes described here were in contrast to those of the first category reported above³ and were of a more marked nature than those described under Paragraph II.

Before concluding this paper it was necessary to investigate whether the phenomenon of paragglutination played any rôle in the observations described above. For this purpose the following experiments were made.

1. The lytic principles employed in this work also contained products of bacteria, at the expense of which the phages were regenerated. It was decided to determine whether bacteriophage-free filtrates of these cultures would not induce similar changes. Filtrates of 1 week old

³ Page 154.

Rb streptococcus and *B. coli* 42 were used. Several passages of 55, 108 and V strains were made in phosphate broth containing 1 to 10 dilutions of Rb streptococcus filtrate. Rb streptococcus was passed through *B. coli* 42 filtrate. Agglutination reactions of the cultures prepared in this manner with homologous normal culture sera did not differ from those obtained with homologous cultures. It thus became

TABLE VI.

The Influence of SL Phage upon Agglutination of V Strain.

Sera	Absorbed by strain	Agglutination before absorption and percentage of absorption	
		V	VSL
XVII	=	3200	0
XVII	V	100 per cent	—
XVII	V SL	0 per cent	—
XIX	=	0	5120
XIX	V	—	0 per cent
XIX	V SL	—	100 per cent

XIX, V SL strain serum.

TABLE VII.

The Effect of SL Phage upon Agglutination of H.S. Strain.

Sera	Absorbed by strain	Agglutination titer before absorption and percentage of absorption	
		H.S.	H.S./SL
XX	=	0	1280
XX	H.S.	—	0 per cent
XX	H.S. SL	—	100 per cent
XXI	=	5120	0
XXI	H.S.	100 per cent	—
XXI	H.S. SL	0 per cent	—

XX, H.S. SL phage serum; XXI, H.S. serum.

apparent that the phenomenon of paragglutination was not responsible for the changes described in this paper. This belief was further strengthened by the following additional observations.

2. Three animals immunized with SL and C/2 lytic principles failed to show agglutinins for the bacterial strains from which these principles were derived.

3. Sera prepared with SL phage cultures of various strains cross-agglutinated with Rb streptococcus only in those cases in which the normal culture sera cross-agglutinated as well.

4. Various strains of streptococci (erysipelas and scarlet fever groups) which normally cross-agglutinated with Rb streptococcus failed to do so when passed through SL phage.

5. Rb C/2 and V strains were not agglutinated by the anti-C/2 serum.

6. Rb C/2 strain serum failed to agglutinate *B. coli* 42.

CONCLUSIONS AND SUMMARY.

A series of experiments was carried out on various strains of streptococci in order to ascertain the changes which bacteriophage produces in the phenomena of agglutination of these organisms. The results can be placed in the following categories.

1. Loss of specific agglutinability was observed with partial and with what appeared as complete loss of specific agglutinin absorption and agglutinogenic properties.

2. Partial modification of the antigen, bringing about inagglutinability of the strains with complete preservation of agglutinogenic and agglutinin-absorption properties. Coincidentally, additional components appeared. Some of these were related to the specific antigens of the organisms from which they were derived, while others were not. In two instances these components were of "group" character.

3. "Complete" modification observed with two strains consisting of complete transformation of the normal culture agglutinogens into antigens of an entirely different specificity.

It appears from these studies that the bacteriophage phenomenon may play an intricate rôle in serological grouping of various strains of pathogenic streptococci.

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ETIOLOGY OF OROYA FEVER.

IX. BACTERIUM PERUVIANUM, N. SP., A SECONDARY INVADER OF THE LESIONS OF VERRUGA PERUANA.

By HIDEYO NOGUCHI, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

PLATE 6.

(Received for publication, November 3, 1927.)

In the course of an experimental investigation of Carrion's disease, at the stage when bacteriological and experimental study of the characteristic lesions of verruga peruana was desirable, I was so fortunate as to obtain, through the kindness of Professor Oswaldo Herccelles, of Lima, two subcutaneous nodules, excised under aseptic conditions, from two verruga patients (Cases P 5 and J 45), and forwarded in separate sealed tubes in the ship's refrigerators from Lima to New York. A strain of *Bartonella bacilliformis* was isolated from one of the nodules (Case P 5), as has been reported elsewhere,¹ but another microorganism was also present in the tissue, and was isolated in pure cultures in both instances. This organism is particularly interesting because of its striking morphological similarity to *Bartonella bacilliformis* and its marked difference from this parasite in cultural and pathogenic properties.

Cultural Properties.

The microorganism in question grows rapidly on ordinary bacteriological culture media at 37°C. as well as at 25°C., the latter temperature being the optimum one. Round, discrete, elevated, grayish, translucent colonies measuring 1 to 2 mm. in diameter make their appearance on a plain or blood agar plate within 24 hours. They reach a size of 2 to 4 mm. within several days but seldom become much larger. The colonies are spherical, and the margin smooth; in consistency they are somewhat firmer than colonies of *B. coli* or *B. proteus*, and they show no

¹Noguchi, H., *J. Exp. Med.*, 1927, xlv, 175.

tendency to spread over the surface of the medium. In 7 to 10 days at room temperature a trace of brownish tint appears in colonies on the surface of agar. On blood agar plates hemolysis takes place and gradually extends to a considerable distance around the colonies. A characteristic pungent odor is noticeable when the plate is uncovered. Broth is diffusely clouded by the growth of the organism, and within a few days a delicate grayish pellicle is formed around the wall of the culture tube at the top of the fluid and a fluffy sediment at the bottom. As the culture grows older the broth becomes markedly mucilaginous. No putrefactive or other odor is produced either in broth or on plain agar. Löffler's serum undergoes slow but complete liquefaction within about 2 weeks at 25°C. No carbohydrates are fermented by the organism, and no hydrogen sulfide is produced on any medium. Cultures remain transplantable for a period of at least 3 months if kept constantly at 25°C. No growth takes place under strictly anaerobic conditions.

Morphology.

Individuals in young colonies on an agar surface are actively motile and measure about 0.3 to 0.4 μ in width and 0.6 to 1.2 μ in length. In older cultures longer forms, 2 to 4 μ , are present. When grown on a blood agar slant or on leptospira medium the organisms are somewhat thinner (0.2 to 0.3 μ in width) and longer than the young forms on an agar surface and give the impression of delicate slender rods (Fig. 3). They are less actively motile and may be clumped into masses of many individuals (Fig. 1) which resemble to some extent the characteristic masses of *Bartonella bacilliformis*. Forms grown on broth are of medium size and remain actively motile for many days. In cultures several days old the motile organisms are intermingled with small oval or coccoid forms which may be in pairs or short chains. Except for occasional very long individuals, which may be bent and thickened at one end, the organisms are as a rule straight and have pointed ends. No bifid or branching individuals have so far been noted.

The organism is Gram-negative and stains fairly well with fuchsin or methylene blue. Brief staining in Giemsa's solution gives it a lavender color; with more prolonged staining it becomes reddish. The general aspect of the organism in stained smears (Figs. 1 and 2) resembles that of *Bartonella bacilliformis* in similar preparations, but the individual organisms are more sharply defined than are the individual bartonellas, owing perhaps to a greater affinity for the dyes. No tendency to bipolar staining has been noticed.

One of the striking features of the organism is its characteristic spirally curved flagella (Fig. 4) which are present at one end of the body and may be two, three, or four in number. So far as I am aware, the only other pathogenic organism having flagella of this type is *Bartonella bacilliformis*.

Because of its presence in material derived from Peru, the organism has been named *Bacterium peruvianum*.

Pathogenicity.

When *B. peruvianum* is inoculated intradermally into monkeys (*Macacus rhesus*, *Cercopithecus callitricus*) or dogs, the sites of inoculation become edematous, congested, and necrotic within a few days, and open ulcers with raised margins finally result; these usually heal within 10 to 14 days. The Strain P 5 was more actively pathogenic than the J 45. Unbroken nodules such as were occasionally produced by the P 5 strain resolved after about 10 days.

Cultures made from the blood of the animals during the height of the local reactions remained sterile in all instances; there was no invasion of the general circulation by the organism. Whether or not the injection of *B. peruvianum* into the blood circulation in large quantities will result in fatal septicemia has not been determined.

B. peruvianum induces an acute fatal septicemia in rabbits, guinea pigs, rats, and mice, when administered intravenously or intratesticularly. A broth culture 48 to 72 hours old was lethal within 24 to 48 hours in a quantity of 0.1 to 1 cc. Autopsy reveals very striking and characteristic changes in the abdominal viscera.

The liver seems to be most affected; it is somewhat enlarged, tense, uniformly peppered with innumerable minute, intensely red spots which make the whole organ appear red. The stomach shows many diffuse and punctiform hemorrhages recognizable through the congested serosa. The entire intestine appears diffusely limpid red. The kidneys are swollen and congested, and the adrenals are much enlarged and deep red. The spleen is not noticeably enlarged but is soft and extremely dark bluish red. The peritoneal cavity contains some limpid pink fluid. No fibrinous exudate was found on the surface of any organ. The abdominal muscles are frequently spotted with hemorrhagic areas of moderate size. The lungs are congested but show no hemorrhages. The heart is flabby, but there is no pericarditis or pleurisy. *B. peruvianum* can be recovered in pure culture from heart's blood or spleen.

When inoculated intratesticularly, the animals succumb within 1 to 3 days and show the same changes as do animals injected intravenously. The scrotum and testicle both show intense edema, congestion, hemorrhages, and necrosis.

There was no intercurrent infection with *B. lepticus* or *B. monocytogenes*² in any of the animals.

Differentiation of B. peruvianum from Other Pathogenic Microorganisms.

The characteristics of *B. peruvianum* may be summarized as follows: Proper motility by means of multiple unipolar flagella; non-retention of Gram's stain; strict aerobiosis; better growth at 25°C. than at 37°C.; presence of proteolytic and absence of sugar-splitting ferment; mucin production in broth; hemolytic action; and a wide range of characteristic pathogenic properties for rabbits, guinea pigs, rats, mice, dogs, and monkeys. No microorganism having been previously described which corresponds with the foregoing description, the organism in question is regarded as a new species.

Bartonella bacilliformis resembles *B. peruvianum* in morphology, in its inability to ferment carbohydrates, in growing better at 25°C. than at a higher temperature, in not retaining Gram's stain, in being strictly aerobic, and in having one to four unipolar flagella. The two organisms differ widely in other respects, however. *Bartonella bacilliformis* is unable to grow on any fluid medium so far tried, or any medium which does not contain blood elements; it grows very slowly, and the colonies are very minute; it lacks proteolytic or hemolytic activity, it is difficult to disperse into a homogeneous suspension, and above all, it has specific pathogenic properties. Moreover, a potent anti-*bacilliformis* immune serum gives a specific complement fixation and a distinct agglutination reaction with *Bartonella bacilliformis*, but none with *B. peruvianum*.

B. proteus and allied organisms are distinct from *B. peruvianum* in morphological and cultural properties. The flagella of the *proteus* group are peritrichal, and hydrogen sulfide is produced by the organisms in the course of growth.

The *coli-enteritidis-typhoid* group ferments carbohydrates, and does not liquefy Löffler's serum. The flagella of this group are peritrichal.

² Murray, E. G. D., Webb, R. A., and Swann, M. B. R., *J. Path. and Bact.*, 1926, xxix, 407. Pirie, J. H. H., *Pub. South African Inst. Med. Research*, 1927, iii, 185. I am indebted for cultures of this microorganism to Dr. J. C. G. Led-ingham, Director of the National Collection of Type Cultures at the Lister Institute.

B. monocytogenes has a single polar flagellum and the property of fermenting a great many carbohydrates.

*Bacillus rickettsiformis*³ resembles *Bacterium peruvianum* in some respects, in morphological features, in growing best at low temperatures, and in producing mucin in broth cultures. But the *rickettsiformis* is non-pathogenic and ferments several sugars.

SUMMARY.

A minute, pleomorphic, motile, Gram-negative bacterium has been isolated from two specimens of nodular tissue from human verruga. In films and sections of the original tissues the organism in question is difficult to distinguish from *Bartonella bacilliformis*, with which it was associated, and even in pure culture it has a number of properties in common with that parasite. No sugars are fermented by it, it is an obligate aerobe, the optimum temperature for its growth is 25°C., and it has two to four spiral flagella attached to one end of the body. It is, however, readily cultivated on any ordinary culture medium. Broth cultures contain much mucin, but no hydrogen sulfide is formed. Coagulated serum is liquefied by its growth, and the red corpuscles in a blood agar plate are hemolyzed.

Rabbits, guinea pigs, rats, and mice develop acute, fatal septicemia as a result of intravenous or intratesticular inoculation of young cultures. The liver is characteristically affected and shows a general parenchymatous degeneration and necrosis; the entire gastrointestinal tract is intensely congested, and numerous hemorrhagic areas are present; the spleen, dark and soft, is rarely much enlarged; the kidneys are swollen and congested; the adrenals are much swollen and intensely red; the lungs are sometimes congested but otherwise normal. In the case of intratesticular inoculation the scrotum and testicle both undergo rapid gangrene. In monkeys no septicemia has been observed, but a violent local reaction—swelling, congestion, sometimes necrosis—follows intradermal inoculation.

Since no microorganism corresponding in character with this one has previously been described, it is regarded as a new species, and because of its presence in material obtained from Peru it has been given

³ Noguchi, H., *J. Exp. Med.*, 1926, xliii, 515.

the name *Bacterium peruvianum*. The significance of the association of *B. peruvianum* with *Bartonella bacilliformis* deserves further investigation; it is not impossible that the two organisms are introduced into the human body by the same blood-sucking insect.

Cultures of *Bacterium peruvianum*, n. sp., an organism associated with *Bartonella bacilliformis* in verruga tissues.



FIG. 1.

Giemsa's stain. $\times 1000$.

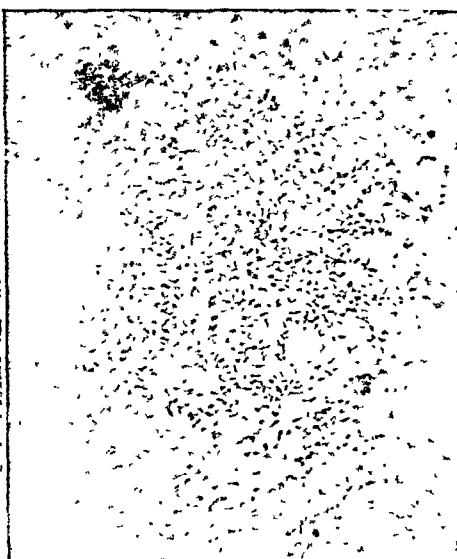


FIG. 2.

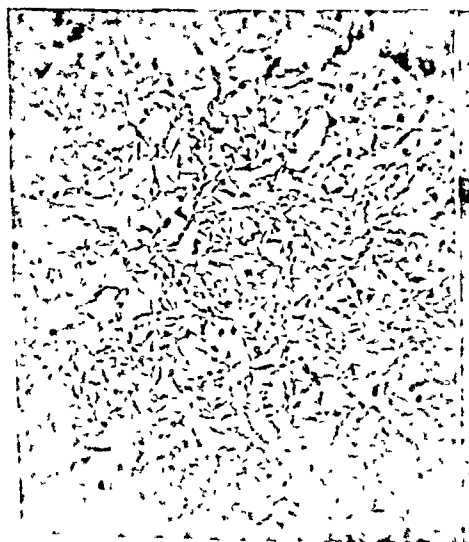


FIG. 3.

Giemsa's stain $\times 1000$.

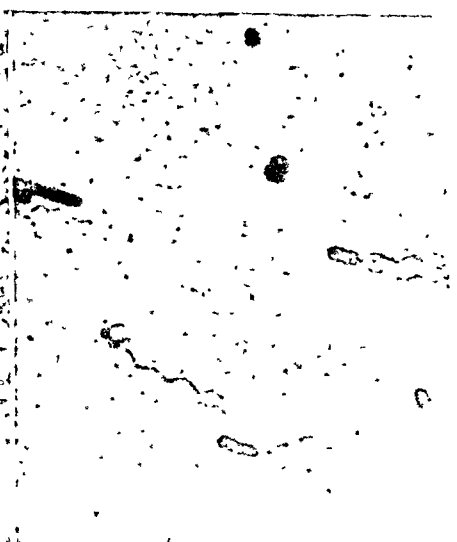


FIG. 4.

Ziehl-Neelsen flagella stain. $\times 2000$.

ON PRECIPITABLE SUBSTANCES DERIVED FROM BACILLUS TYPHOSUS AND BACILLUS PARA- TYPHOSUS B.

BY J. FURTH, M.D., AND K. LANDSTEINER, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

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The present study was primarily undertaken in order to verify certain findings reported on the extraction of precipitable substances of *B. typhosus* with alcohol and ether, but it was extended later to a more general investigation of the precipitable substances of this and allied microorganisms (1).

A number of papers have dealt with the question of lipoids or alcohol-soluble specific substances of typhoid bacilli.

Pick (2) investigating chemically the nature of the specific substances of the *B. typhosus* described one soluble in alcohol which gave no protein and no Molisch reaction and resisted digestion. In concentrated solution it was precipitated by immune sera. Nicolle (3) reported that the agglutinogens of typhoid bacilli are soluble in alcohol and ether but Winterberg (4) was unable to confirm these results. That the lipoids of typhoid bacilli, or even the saponified fats, possess antigenic activity was claimed by Schachenmeier (5) and Stuber (6). Borcic (7), H. Schmidt (8), and Weil and Felix (9), however, failed in their attempts to use alcohol or ether extracts of the typhoid bacilli for immunization. Similar negative results were recorded by Zurugzoglul (10). Very recently Przesmycki (11) has reported that ordinary antibacterial immune sera react specifically with alcoholic extracts of homologous bacilli. Definite statements that bactericidal immune sera develop after the injection of alcohol and ether extracts of typhoid bacilli have been made by Schlemmer (12). So far as we are aware Schlemmer's work has not been repeated as yet.

EXPERIMENTAL.

Bacillus typhosus.—Immune sera were produced by injections of ether extracts of typhoid bacilli according to the directions of Schlemmer. The sera obtained had very weak agglutinating and bactericidal activity when compared with typhoid immune sera obtained in the usual way with whole bacilli.

Attempts to prepare alcohol-soluble precipitable substances from typhoid bacilli by the method of Pick gave no clear-cut results with our material. A saline extract of agar cultures of *B. typhosus* was precipitated with alcohol and both the centrifuged sediment and the supernatant fluid were tested with a precipitating immune serum. It was found that the bulk of the precipitinogen was carried down in the precipitate while the solution contained only a small fraction of the active substance.

Definite results were secured when in place of ether or absolute alcohol 75 per cent alcohol was used for the extraction, in the manner already described for *Vibrio cholerae* (13).

B. typhosus was grown on agar for 24-48 hours at 37°C. The bacilli were centrifuged, washed once with saline and twice with 95 per cent alcohol, and heated for about 2 hours with absolute alcohol (10 cc. per Blake bottle) on the steam bath. The bacilli were collected by filtration on a hot water funnel and extracted twice with boiling 75 per cent alcohol for about 2 hours, the suspension filtered hot, and the filtrate chilled in the ice box. The precipitate formed was dried after washing with 95 per cent alcohol and absolute alcohol and ether. For the precipitin and chemical tests the substance was dissolved in weak alkali and precipitated by acidifying with acetic acid. The procedure was repeated two to three times. This preparation will be referred to as 75 per cent alcohol extract or P1.

When the substance was tested with ordinary typhoid immune sera obtained from rabbits injected with typhoid bacilli heated to 60-62°C. for about 40 minutes, faint reactions only were noticed. We tried therefore to prepare immune sera with the product itself. It was found to possess strong antigenic activity. Two to three injections of 0.2-2 mg. each were sufficient for the production of immune sera which, in contrast to the ordinary typhoid immune sera, precipitated strongly the extracted substance, but agglutinated only feebly suspensions of *B. typhosus* (Table I).

The substance is insoluble in water but soluble in weak alkali. It precipitates out of its solution on acidifying. It gives strong protein reactions. After digestion with trypsin or treatment with antiformin the substance is no longer precipitated by immune sera. On hydrolysis for 5 hours only slight or no reduction of Fehling's reagent was found. The product contains only traces of phosphorus

and traces of lead-blackening sulfur. The N content of the ash-free preparation was 15.7 per cent.

Since the alcohol extract gave definite reactions only with a special immune serum the attempt was made to prepare solutions which would react with the ordinary immune sera. Such active solutions have been obtained previously by various means of extraction, as with saline solution (Pick (2), Weil and Felix (9)), dilute alkali (Zinsser and Parker (14)), antiformin (Altmann and Schultz (15), Krumwiede and Nobel (16)) etc.

We extracted active substance in the following manner:

Washed bacilli previously treated with 95 per cent, hot absolute, and hot 75 per cent alcohol, as described above, were extracted for 2 hours on the steam bath with 2 cc. of .9 per cent saline solution per Blake bottle. After centrifugalization the solution was precipitated with about half the volume 95 per cent alcohol. This crude material was purified to a certain extent by repeated precipitations with acid, and by extraction with 75 per cent alcohol as described below. The product obtained is designated as P2.

Immune sera active for P2 were got by immunization of rabbits with the bacillary residue remaining after the preparation of P1. These sera had a considerable agglutinin titer for live typhoid bacilli.

The product P2 dissolves readily in water and is precipitated by acidulating. It has the reactions and composition of a protein (N 17.2 per cent) and contains only faint traces of phosphorus and traces of lead-blackening sulfur. After hydrolysis for 5 hours only a slight or no reduction of Fehling's reagent was found.

By digestion with trypsin or treatment with antiformin the serological activity, tested by precipitation, was destroyed like that of P1.

That specific substances differing from those just described are present in typhoid bacilli seemed to follow from the important study of Douglas and Fleming (17, *cf.* 11). These authors digested with trypsin typhoid bacilli previously extracted with acetone. Using this material they produced in rabbits immune sera which reacted with the solution resulting from the digestion after removal of an undigested part. The sera had a high bactericidal activity but a weaker agglutinating activity than ordinary immune sera and precipitated more intensely the soluble digestion product. The authors

seem inclined to believe that this precipitable substance is a lower degradation product of proteins.

On repeating¹ the experiments of Douglas and Fleming which deal with the precipitinogen we arrived at confirmatory results. The immune sera obtained had moderate agglutinating power (2000–5000) and only a slight precipitating action for the substances P1 and P2. In conformity with these facts the immune sera for P1 and P2 did not react with the solution resulting from digestion.

When the solution resulting from digestion was made strongly alkaline and precipitated with alcohol a material was obtained with a high content in carbohydrates. Substances with similar properties could be prepared by various methods, *e.g.* as follows:

Typhoid bacilli grown on agar were taken up in N/2 sodium hydroxide solution and kept for about 1½ hours at 37°C. After adding hydrochloric acid till only a slight alkalinity remained and centrifuging, the fluid was precipitated with 1½ volumes of alcohol, the precipitate was redissolved, and after removing some insoluble material and adding hydrochloric acid to make the solution N/10 it was again precipitated with 1½ volumes of alcohol.

This crude product, designated as C_{typh.}, gave negative or faint protein reactions, yielded 39.4 per cent reducing sugars after hydrolysis for 5 hours with N/2 hydrochloric acid, and contained N 4.05 per cent (ash-free). Qualitative tests for phosphorous were strongly positive. (A sample obtained after precipitation with barium hydroxide gave, on hydrolysis, about 46 per cent reducing sugars and had a N content of 3.9 per cent.)

Apparently the same substance can be extracted by dissolving the typhoid bacilli with alkaline hypochlorite solution as suggested by Altmann and Schultz, Krumwiede and Nobel.

Typhoid bacilli grown on agar were suspended in saline, and antiformin was added in sufficient amount to cause solution of the bacteria at 55°C. When to this unneutralized solution alcohol was added an active substance separated out. This was suspended in water and after removal of some insoluble material again precipitated with alcohol.

The preparation C_{typh.} exhibits serological reactions similar to those of the substance of Douglas and Fleming. Its serological activity,

¹ In place of acetone, alcohol was used for the extraction.

unlike that of P1 and P2, is not affected by tryptic digestion and treatment with antiformin (1-10 per cent solution for 30 minutes at 37°C.). The preparation was not precipitated in as high dilutions as apparently similar substances from other bacilli.

The precipitation tests performed with the preparations of *B. typhosus* described are summarized in Table I.

TABLE I.

Precipitation Tests with Various Preparations of B. typhosus.

Preparation	Dilution of antigen	Immune sera prepared with					Normal serum
		Whole bacilli		P1	Bacilli after extraction of P1	Digested bacilli	
		96	31				
P1	1,000	+±	tr.	+++	f.tr.	+	f.tr.
	5,000	tr.	0	++±	tr.	±	0
	25,000	0	0	+	0	0	0
	100,000	0	0	±	0	0	0
	500,000	—	—	0	—	—	—
P2	1,000	+		±	++	tr.	0
	5,000	+		tr.	+++	0	0
	25,000	±		0	++	0	0
	100,000	tr.		0	+	0	0
	500,000	0		—	tr.	—	—
C _{typh.}	1,000	++		0	0	+++	0
	5,000	+		0	0	++	0
	25,000	tr.		0	0	±	0
	100,000	f.tr.		0	0	tr.	0
	500,000	0		—	—	f.tr.	—
Agglutinin titer for live bacilli		50,000	20,000	100	10,000	2,000	100 neg.

In this and the following experiments 1 drop of immune serum was added to 0.2 cc. of the diluted antigen and the reactions were read after 2 hours incubation at room temperature.

It seems from these experiments that the three preparations tested are distinctly different as to their serological reactions. This holds particularly for substance C. Between P1 and P2 group reactions occur to a slight degree and both react weakly with an immune serum for C. Since this latter reaction did not disappear after treatment

with antiformin it is very probably due to the presence of C in P₁ and P₂. With several other preparations the group reactions were more pronounced.

When preparing P₁ the bacilli were extracted several times with 75 per cent alcohol. The first and second extracts reacted mainly with sera for P₁ whereas later extracts gave also considerable reactions with sera for C. In preparing P₂ a similar behaviour was noted. The second saline extract gave a stronger reaction for C than the first. The content in C of the protein preparations could be reduced by repeated precipitations with acid. P₂ could be freed to a certain extent from P₁ by dissolving in 1 per cent saline solution, adding alcohol to a concentration of 75 per cent, boiling, and filtering hot. P₂ was isolated from the insoluble part by dissolving in slightly alkaline water and precipitating with acid.

Bacillus paratyphosus B.—From *Bacillus paratyphosus B* a specific substance was prepared by extraction with 75 per cent alcohol as described above. This preparation contained proteins and a considerable amount of carbohydrates. It was precipitated strongly by the ordinary paratyphoid B immune sera and induced the formation of agglutinins and precipitins when injected into rabbits. It was precipitated to a certain extent by immune sera for the substance P₁ of typhoid bacilli, but this reaction disappeared after digestion with trypsin or treatment with antiformin while the reaction with paratyphoid immune sera persisted after such treatment. This behaviour suggested the presence in the alcoholic extract of two specific substances analogous to the preparations P₁ and C from *B. typhosus*. In order to prepare the latter the following method was used.

Bacilli previously extracted with hot absolute alcohol were heated on the steam bath with saline solution for about 1½ hours. The suspension was centrifuged, acidulated, and after the removal of the precipitate by centrifugalization the supernatant liquid was precipitated with alcohol. The precipitation with alcohol was repeated 2-4 times in acid and in alkaline solution.

The substance which will be designated as C_{paratyph. B} gave only faint protein reactions. After hydrolysis with N/2 hydrochloric acid for 5 hours 63.8 per cent reducing sugar was found, calculated as glucose. An analysis gave the following figures for ash-free substance: C 43.8 per cent, H 6.5 per cent, N 1.86 per cent, P 2.06 per cent, ash 7.16 per cent. The yield was considerable and amounted to 10-20 mg. per Blake bottle.

Tests for Species Specificity.

The precipitable substances derived from typhoid and paratyphoid bacilli and the corresponding immune sera were tested with a number of other immune sera and antigens respectively (Tables II, *a*, *b*, and III).

TABLE II, *a*.

Precipitation Tests with P1 from B. typhosus and Various Immune Sera.

Dilutions of antigens	Immune sera obtained with								
	<i>B. typhosus</i>		<i>B. enteritidis</i>		<i>B. paratyphosus B</i>		<i>V. cholerae</i>		<i>Proteus</i> HX19
	Whole bacilli	75 per cent alcohol extract	Whole bacilli	75 per cent alcohol extract	Whole bacilli	75 per cent alcohol extract	Whole bacilli	75 per cent alcohol extract	Whole bacilli
1,000	+	tr.	—	f.tr.	±	—	tr.	0	0
5,000	tr.	0	+++	0	f.tr.	+++	f.tr.	0	0
50,000	0	0	+	0	0	+	0	0	0
500,000	0	0	0	—	—	0	—	—	—

TABLE II, *b*.

Precipitation Test of an Immune Serum for P1 and Crude 75 Per Cent Alcohol Extracts from Various Organisms.

Dilutions of antigens	75 per cent alcohol extract obtained from					
	<i>B. typhosus</i>	<i>B. enteritidis</i>	<i>B. paratyphosus B</i>	<i>B. coli</i>	<i>V. cholerae</i>	<i>Proteus</i> HX19
1,000	—	—	—	+	0	0
5,000	+++	++	+	+	0	0
50,000	+	±	tr.	tr.	0	0
500,000	0	0	0	—	—	—

It appears from Table II, *a* and *b*, that the serum against the substance P1_{typh.} gives marked group reactions with the analogous substances of *B. enteritidis*, *B. paratyphosus B*, and *B. coli*, and not with the preparations of the more distant organisms, *V. cholerae* and *Proteus* HX19. This result was confirmed by tests with various immune sera.

There were less pronounced group reactions with the preparation

TABLE III.
Precipitation Tests for Specificity with the Preparations C.

Substances obtained from	Dilutions of antigens	Immune sera prepared with					<i>V. cholera</i> whole bacilli
		<i>B. typhosus</i> digested bacilli	<i>B. enteritidis</i> G whole bacilli	<i>B. paratyphosus</i> B whole bacilli	<i>Proteus</i> HX19 whole bacilli		
<i>B. typhosus</i>	1,000	++±	+	0	0	0	0
	5,000	+±	±	0	0	0	0
	25,000	±	f.tr.	0	0	0	0
	100,000	tr.	0	0	0	0	0
	500,000	0	(10,000)	(1,000)	0	0	0
<i>B. enteritidis</i> G	1,000	++	±	0	0	0	0
	5,000	+±	+	0	0	0	0
	25,000	±	+±	0	0	0	0
	100,000	0	+	0	0	0	0
	500,000	0	±	(5,000)	(100)	0	0
<i>B. paratyphosus</i> B	1,000	+±	0	+±	+±	0	0
	5,000	tr.	tr.	±	+±	0	0
	25,000	0	±	+	+±	0	0
	100,000	0	tr.	+	+±	0	0
	500,000	(1,000)*	f.tr.	±	tr. +±	0	0

<i>Proteus</i> ON19	1,000	0	tr.	0	0	0	+	+	+	+	+	+	0
	5,000	0	0	0	0	0	+	+	+	+	+	+	0
	25,000	0	0	0	0	0	+	+	+	+	+	+	0
	100,000	0	0	0	0	0	+	+	+	+	+	+	0
	500,000						+	+	+	+	+	+	
(2,000)													
<i>V. cholerae</i>	1,000	tr.	tr.	0	0	0	0	0	0	+	+	+	+
	5,000	0	0	0	0	0	0	0	0	+	+	+	+
	25,000	0	0	0	0	0	0	0	0	+	+	+	+
	100,000	0	0	0	0	0	0	0	0	+	+	+	+
	500,000									tr.	tr.	tr.	tr.
(20,000)													

First reading after 2 hours room temperature, the second after standing overnight in the ice box. The figures indicate the agglutinin titre.

* Very weak reactions also in high concentrations of serum.

P2. P2_{typh.} was not acted upon in the dilutions tested by two common immune sera against *B. enteritidis* and gave a weak reaction with only one of two common immune sera against *B. paratyphosus* B. A moderate reaction became evident when a serum very active against P2_{typh.} was tested with a crude P2 preparation of *B. paratyphosus* B, and a faint reaction occurred with a similar preparation of *Proteus* HX19.

The reactions involving the species specificity of the substances C are summarized in Table III. The substance C of *V. cholerae* has already been described (13). C_{OX19} was obtained in a similar manner from *Proteus* OX19.² This latter product gave negative or faint protein reactions and had a carbohydrate content of 57.3 per cent after hydrolysis for 5 hours. A substance apparently with the same serological and chemical properties could be prepared from *B. proteus* HX19 with about the same yield as that got from *Proteus* OX19. C of *B. enteritidis* was prepared in the same manner as C_{typh.} The yield of reducing sugars after hydrolysis of this substance was 56.89 per cent (calculated as glucose for ash-free substance).³

In tests with the substances C showing strong reactions, the precipitates appear generally in heavy flakes or membranes unlike the more fluffy precipitates of proteins as has already been observed by Avery and his coworkers.

DISCUSSION.

The investigations reported may be regarded as an initial step in the study of the antigens of typhoid bacilli and related organisms. The work should be extended in various directions with special reference to the purification of the isolated products, and the rôle they play in the group reactions of the related species. Methods of preparation should be studied, a search made for additional active substances, and various strains of the same organism examined.

The active substances thus far prepared fall into two groups differentiated sharply by their behaviour towards trypsin and alkaline

² A report on specific polysaccharides in *Proteus* HX19 has been made recently by Przesmycki (18).

³ An antigenic solution of *B. enteritidis*, containing traces of proteins and much carbohydrate was dealt with recently by Branham and Humphreys (19).

hypochlorite solution (antiformin). Those of one group (P) are easily destroyed by these agents and behave in general like proteins, while the others (C) are resistant to trypsin and considerably so to antiformin and yield a large quantity of reducing sugar on hydrolysis. There was no definite evidence in our studies with *B. typhosus* of the existence of lipoids as assumed by Schlemmer and others.

The presence in bacilli in general and in typhoid bacilli especially of specific non-protein substances which do not induce antibody formation has been emphasized by Zinsser and Parker (14). An indication of the existence of such products can be found in the paper of Pick although he considered the possibility that his substances were disintegration products of proteins. A similar view was held by Douglas and Fleming (17) concerning their substances obtained by digestion.

In the light of the well known studies of Avery and Heidelberger it seemed likely that the specific component in the products of the above authors may belong to the group of specific carbohydrates, a view substantiated by our findings. The immune sera obtained by us following the directions of Douglas and Fleming reacted on our substance C which is almost free from proteins and rich in carbohydrates. This preparation C will probably prove to be a hapten in our terminology, *i.e.*, a specific substance devoid of antigenic activity.

Substances belonging apparently to the same group, that is to say yielding much sugar on hydrolysis and practically free from protein, were also found in *B. paratyphosus* B, *B. enteritidis*, and *Proteus* HX19 and OX19.²

On cross-testing the various substances of the group C with the corresponding immune sera the strongest group reaction occurred between *B. typhosus* and *B. enteritidis*, the homologous reactions being more intense. The substance of *B. paratyphosus* B was precipitated by the sera for *B. typhosus* and *B. enteritidis* though not very intensely. It is remarkable that the reciprocal reactions, namely those of the paratyphoid B serum with the substances from *B. typhosus* and *B. enteritidis* were entirely negative. For an understanding of these relations further investigations are required, involving also absorption tests. With the substances and sera of microorganisms not belonging to the typhoid groups negative or only faint reactions took place.

Observations similar to ours were made by Krumwiede and Nobel who found strong reactions of typhoid immune sera with antiformin extracts of *B. pullorum* and *B. sanguinarium* and faint reactions with extracts of *B. paratyphosus* B. Probably the active substances in the antiformin extracts correspond to our preparations C.

The tests with the preparations P1 and P2 show that the proteins of a given bacillus may be sharply differentiated by serological reactions in the same way as the proteins of an animal species (20) or those of yeasts (21). No effort has yet been made to fractionate our active protein preparations or to isolate other precipitable proteins.

The high antigenic power of the bacterial proteins is remarkable (22) and it may serve to explain some of the statements published on the antigenic activity of preparations from bacilli that are apparently protein-free. In view of the antigenic capacity of P1 it seems significant that the immune sera formed after injection of whole bacilli heated to 60 or 100°C. have ordinarily only a weak action on P1, so that it is necessary to immunize with the substance itself if one desires to obtain potent precipitins for P1. This phenomenon could be explained either by a change of the precipitable substance through the action of alcohol, or more readily by the masking of its antigenic activity in the original complex. To such an effect one may ascribe also the fact that the sera active for the various precipitable fractions, especially the P1 serum, are considerably less agglutinative than the common immune sera. Examples of both phenomena, namely of the failure of an immune serum for a complex antigen to react with the components of that antigen and of the failure of a serum for one component to react with the complex were encountered in the study of blood antigens (23). Avery and his co-workers (24) have described similar findings in their work on pneumococci.

By absorbing a common typhoid immune serum with bacilli treated with alcohol a fluid was procured which is supposed to contain the flagellar agglutinins (Smith and Reagh (25)) and indeed agglutinated live typhoid bacilli to a high titer and also suspensions containing flagellæ (Orcutt (26)). The fluid failed to precipitate any of our precipitable substances. Similarly an immune serum for *Proteus*

HX19 after absorption with *Proteus* OX19 did not precipitate the substance C_{x19} although it agglutinated intensely *B. proteus* HX19 (not *Proteus* OX19). Thus there is no proof as yet of a connection of these precipitable substances with the so called "labile" agglutino-gen.

SUMMARY.

Attempts to confirm certain statements that ether-soluble specific substances can be obtained from *B. typhosus* have lead to negative results.

Two serologically active protein substances and another that was non-protein have been separated from *B. typhosus*. The first two are not resistant to tryptic digestion or to treatment with alkaline hypochlorite solution whereas the third resists both. One of the proteins could be extracted with 75 per cent alcohol.

Specific precipitable substances reacting like the non-protein substance of *B. typhosus* and containing large amounts of carbohydrates have been prepared from *B. paratyphosus* B, *B. enteritidis*, and *Proteus* HX19 and OX19. Observations on the serological behaviour of these preparations are described.

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ANNOUNCEMENT

THE SIR WILLIAM OSLER MEMORIAL VOLUME

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The first edition of this significant publication has been exhausted. In response to numerous demands a second impression, numbered consecutively with the first, has now been issued, and the type distributed. The book presents, in addition to all previous matter, articles by Sir E. A. Sharpey-Schafer, Dr. L. G. Rowntree, Dr. P. M. Foshay, and others, with several new illustrations including a fine reproduction in colour of Sargent's "Four Doctors." It is attractively bound in cloth, and contains 684 pages and 105 illustrations. The Forewords by Professor William H. Welch, and Sir T. Clifford Allbutt, are followed by over 130 appreciations and reminiscences by various authors. These are grouped in biographic sequence under the five academic periods of Sir William Osler's career, with introductory articles concerning each period by Dr. N. B. Gwyn (Toronto Period), Dr. F. J. Shepherd (Montreal Period), Dr. C. K. Mills (Philadelphia Period), Dr. L. F. Barker (Baltimore Period), and Sir Humphry Rolleston (English Period). The book is completed by a

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The Journal of Pathology and Bacteriology,
Edinburgh, April, 1927.

THE INFLUENCE OF THE ADRENAL GLANDS ON RESISTANCE.

III. SUSCEPTIBILITY TO HISTAMINE AS A TEST OF ADRENAL DEFICIENCY.*

By W. J. M. SCOTT, M.D.

(From the Department of Surgery, the University of Rochester School of Medicine and Dentistry, Rochester, N. Y.)

(Received for publication, November 17, 1927.)

The participation of the adrenal glands in the non-specific resistance of the animal has been previously shown. The clearest and most significant demonstration of this function is probably found in the case of bacterial intoxications, using either dead bacteria or their soluble toxins. The proper dose of the usual pyogenic organisms, killed by heat, seriously injured none of the operated controls but killed all of the doubly adrenalectomized rats (8). This increased susceptibility of adrenalectomized rats has been verified in the case of standard typhoid vaccine by Marine and Jaffe (4) and for diphtheria toxin by Belding and Wyman (2).

The resistance to such bacterial toxic agents is undoubtedly of major importance in pathological physiology. But, in studying quantitatively this function of the adrenal cortex, biological substances have the distinct disadvantage of variation, while a definite chemical compound with a somewhat similar pharmacological effect would be advantageous as a test substance. With this in view in 1923, while studying the effect of killed streptococci, the action of histamine was investigated in a few instances. Inasmuch as this substance seemed to possess the desired characteristics, the investigation of its effect has recently been studied in detail.

There is very little in the literature about the susceptibility of adrenalectomized animals to histamine. Dale (3) originally found

* Presented in abstract before the American Society for Experimental Pathology, Rochester, New York, April 14, 1927.

that cats, while still in good condition after adrenalectomy, were prostrated and killed by surprisingly small doses of this chemical. This finding was verified by Kellaway and Cowell (6). Banting has recently repeated this observation upon dogs (1). In both of these species, however, adrenalectomy results fatally within a few days. The present study reports the effect of histamine in the sublethal adrenal deficiency of rats.¹

Methods.

The plan of procedure was similar to that used in previous studies (7, 8). Rats (*Mus norvegicus albinus*) were kept in individual cages upon a standard diet and at a constant favorable temperature. After a period of observation they were operated upon,² both adrenals being removed from some, and one adrenal and a mass of fat from the site of the second adrenal were removed from others as a control upon the effect of the operation. The adrenals were removed by the dorsal route as being somewhat simpler than the ventral route used in the first study.

At determined intervals after operation the resistance of the rats to histamine injected intraperitoneally and intramuscularly was tested. The compound used was ergamine acid phosphate (Burroughs Wellcome and Co.) and all the dosages are reported on the basis of milligrams of ergamine acid phosphate crystals per 100 gm. weight of the animal. This contains about one-third of the amount of histamine base. The animals tested in this manner were all active and were eating well. Any showing clinical evidence of adrenal insufficiency were excluded. The chemical was usually given intraperitoneally 14 days after operation.

Results.

The rat is ordinarily very resistant to histamine. I have not determined exactly the minimum lethal dose for my control animals due to the expense of the large amounts required. An operated control rat was apparently recovering from 110 mg. of ergamine acid phosphate per 100 gm. given in two doses 25 minutes apart and required another large dose 2 hours later to kill it. 30 mg. of ergamine acid phosphate per 100 gm. intraperitoneally was not fatal to four control rats and

¹ Since writing this report I have found a statement by Crivellari (Crivellari, C. A., *Am. J. Physiol.*, 1927, lxxxi, 414) that adrenalectomized rats have a greatly increased susceptibility to histamine.

² All operations were carried out under ether anesthesia.

killed one operated control rat that had recently survived an injection of peptone. In fact the amount of histamine that is required to kill normal and operated control rats is enormous. Voegtlin and Dyer (9) reported the minimum lethal intravenous dose of histamine phosphate to be 90 mg. per 100 gm. The mode of administration affects the outcome greatly, and undoubtedly the minimum lethal intraperitoneal dose is larger than when the substance is given intravenously. All of 12 operated control rats survived doses of 10 to 20 mg. of ergamine acid phosphate per 100 gm. For tissue study eight others were sacrificed in excellent condition, obviously recovering from the transient effects of the injection in the same dosage when the doubly adrenalectomized animals died or were moribund. In fact none of the normal or operated control animals were rendered comatose or non-reactive to stimulation by this dose. The maximum effect of it was transient respiratory difficulty and a quieting of the animal.

Of 36 doubly adrenalectomized rats all but two were killed by this dose (10 to 20 mg. per 100 gm.). In 28 of these the drug was given intraperitoneally, with fatal result in each instance. In addition to these seven other adrenalectomized rats, given the same amount of histamine intraperitoneally, were sacrificed for histological study in a moribund condition. The only two doubly adrenalectomized rats to survive 10 mg. per 100 gm. of ergamine acid phosphate were given the drug intramuscularly. Six others injected in the latter manner were killed by 15 mg. per 100 gm. Two of the rats that as controls had survived 10 mg. per 100 gm. were later killed by the same dose after the second adrenal was removed. The exact time intervals after operation, the period of survival and the mode of injection may be determined from the tabulation (Table I). Most of the rats were injected intraperitoneally about 14 days after operation. All that were tested were in good condition and were indistinguishable in appearance and behavior from the operated control rats in adjoining cages. Most of them had lost a little weight after operation (averaging 5 per cent). Such was not the cause of the fatal effect, however, as the latter occurred even when the weight loss was absent.

The striking difference in susceptibility to histamine displayed by the adrenal deficient and the control animals is shown also in the effect of larger doses. Compare the result of injecting 60 mg. per 100

TABLE I.

Effect of Ergamine Acid Phosphate in Adrenalectomized and Control Rats.

Rat		Operation		Injection				Result
No.	Sex	Weight	Removal of adrenals	Interval since operation	Weight	Method†	Dose	Period of survival in hrs.
		gm.		days	gm.		mg. per 100 gm.	
B1	♀	212	Left*	81	212	P	20	Survived
B2	♀	235	Right*	81	250	P	30	24
B3	♀	216	Right*	67	216	P	10	Survived
B4	♀	245	Right*	70	263	P	20	Survived
B5	♂	180	Right*	23	189	P	20	Survived
B7	♂	250	Right*	7	237	P	20	Survived
R2	♂	300	Right*	12	308	P	10	Survived
R3	♀	258	Right*	12	254	P	10	Survived (cf. R3 later)
R5	♀	308	Right*	12	310	P	10	Survived (cf. R5 later)
R8	♀	142	Right*	12	140	P	10	Survived
R13	♂	198	Right and spleen	14	224	M	15	Survived
R18	♂	—	None	—	294	M	30	Survived
R20	♀	184	Right*	16	192	M	15	Survived
R38	♂	220	Right*	4	220	P	60	Recovering when sacrificed after 2 hrs. (cf. with R41)
R39	♂	228	Perirenal fat	4	220	P	10	Survived
R40	♂	240	Right*	4	245	P and M	180	Moribund after 45 min.
B6	♀	—	Both	21	205	P	15	1
B8	♀	216	Both	7	208	P	20	2½
B9	♀	177	Both	7	160	P	5	4½
B10	♀	207	Both	7	205	P	10	1
R1	♂	360	Both	12	324	P	10	35 min.
R3	♀	254	Second	7	230	P	10	2 (cf. R3 above)
R4	♀	218	Both	12	196	P	10	1¾
R5	♀	310	Second	7	302	P	10	2 (cf. R5 above)

* In most of the control operations when one adrenal was removed, the other adrenal was exposed and a piece of fat from its vicinity was removed to reproduce as completely as possible the physical conditions of the bilateral extirpation.

† In this column P signifies intraperitoneal and M signifies intramuscular.

TABLE I—*Concluded.*

Rat		Operation		Injection				Result
No.	Sex	Weight	Removal of adrenals	Interval since operation	Weight	Method	Dose	Period of survival in hrs.
		gm.		days	gm.		mg. per 100 gm.	
R6	♀	174	Both	12	160	P	10	40 min.
R7	♀	272	Both	12	256	P	10	1½
R9	♀	222	Both	12	202	P	10	40 min.
R10	♀	218	Both	12	194	P	10	2
R11	♂	184	Both	12	206	M	10	Survived
R12	♂	208	Both	12	224	M	10	Survived
R14	♂	220	Both	13	216	M	15	2
R15	♂	218	Both	13	212	M	15	2
R16	♂	192	Both	13	190	M	15	1
R17	♀	148	Both	12	138	M	15	25 min.
R19	♀	160	Both	17	154	M	15	2½
R21	♀	144	Both	16	136	M	15	4½
R22	♂	264	Both	14	250	P	10	45 min.
R23	♂	278	Both	14	265	P	10	1½
R24	♂	234	Both	14	205	P	10	1½
R25	♂	250	Both	14	234	P	10	1½
R26	♂	232	Both	14	205	P	10	1½
R27	♂	280	Both	14	260	P	10	1½
R28	♂	242	Both	14	230	P	10	1½
R29	♂	244	Both	14	245	P	10	30 min.
R30	♂	278	Both	14	252	P	10	1
R31	♂	256	Both	14	250	P	10	1½
R32	♂	262	Both	14	255	P	10	45 min.
R33	♂	212	Both	14	220	P	10	1
R34	♂	224	Both	1	—	P	10	1
R35	♂	202	Both	1	—	P	10	50 min.
R36	♂	210	Both	4	200	P	10	40 min.
R37	♂	242	Both	4	235	P	20	25 min.
R41	♂	246	Both	4	230	P	60	2 min. (cf. with R38)

gm. in Rats R38 and R41, both active and in good condition before injection. The operated control animal was sacrificed 2 hours after the injection, apparently recovering from it, while the doubly adrenalectomized rat was killed by the histamine in 2 minutes.

Most doubly adrenalectomized rats, then, are killed by histamine in doses that produce only transient symptoms in operated control

rats and, in my experience, 10 mg. per 100 gm. of ergamine acid phosphate injected intraperitoneally has regularly proven fatal, if given in the 2nd week after bilateral adrenalectomy.

DISCUSSION.

As the first corollary of this work I propose the reaction of rats to histamine injected intraperitoneally in the 2nd week after operation as one test of adrenal deficiency. While we have not directly proven that the function demonstrated in this diminished resistance to histamine is cortical, from its results viewed in the light of certain previous investigations this conclusion appears to be justified. Kellaway and Cowell (6) correlated the fatal effect of small doses of histamine in their operated cats with the destruction of the cortex and not with the loss of the medulla. Banting (1) found that adrenalin could not counteract the fatal effect of minute doses of histamine for adrenalectomized dogs. And Jaffe (5) demonstrated that autoplasmic cortical transplants increase the diminished resistance of adrenalectomized rats to the toxic effect of killed bacteria. Thus, in correlating these findings with our data it appears that the function involved is cortical and is not related to adrenalin deficiency.

Physiological studies of the adrenal cortex have been handicapped by the fact that there has been no definite simple criterion of its function in any but the terminal phases. This has made extremely difficult the important search for a substitution product to compensate for lost cortical function. Changes in the blood chemistry of these animals, while striking in the terminal stages, are not sufficiently delicate or constant in the period of well being of the animal to serve as an index of the effect of any procedure upon the adrenal deficiency. While less desirable than a chemical index that could be followed in the same animal, the reaction of adrenalectomized rats to histamine offers a method of determining the substitutive effect of a procedure for that adrenal function which influences the non-specific resistance of the animal. At the present time this test is being used in a study of the efficacy of adrenal cortex extracts.

During the course of this study interesting abnormal responses on the part of the peripheral vascular system in adrenal insufficiency have been observed. These will be reported in a separate paper.

CONCLUSIONS.

1. The resistance of rats to histamine is greatly diminished after adrenalectomy.

2. This susceptibility to histamine is proposed as a functional test for deficient adrenal cortex function.

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STUDIES IN THE COMMON COLD.*

II. A STUDY OF CERTAIN GRAM-NEGATIVE FILTER-PASSING ANAEROBES OF THE UPPER RESPIRATORY TRACT.

BY KATHERINE C. MILLS, G. S. SHIBLEY, M.D., AND A. R. DOCHEZ, M.D.
(From the Department of Medicine of the College of Physicians and Surgeons, and
the Presbyterian Hospital, New York.)

(Received for publication, November 11. 1927.)

The common cold is generally conceded to be of infectious origin. Attempts to assign the causative rôle to the bacteria more readily found in the upper respiratory tract during infection or in health have been uniformly unsuccessful. Such organisms have included, *Micrococcus catarrhalis*, *B. Pfeifferi*, pneumococcus, streptococcus, staphylococcus, diphtheroids, certain anaerobes and various other bacteria. It is perfectly clear that several of these, such as, hemolytic streptococcus, *B. Pfeifferi*, *Staphylococcus aureus* and pneumococcus, play an important part in the later phases of infection or in complications, but the actual inception of the cold would seem to be due to an organism or organisms as yet unknown. Much favorable evidence has been put forward (chiefly by transmission experiments) to implicate a filter-passing virus (Kruse (1), Foster (2), Olitsky and McCartney (3)); other observers, however, have obtained unconvincing results (Schmidt (4), Williams *et al.* (5), Branham and Hall (6), Robertson and Groves (7)).

Olitsky and McCartney (3), in their work with cold cultures, grew certain filter-passing anaerobes, previously described by Olitsky and Gates (8), to which they preferred to assign no etiological rôle in view of their occasional appearance in healthy normal individuals.¹ It was hoped that a careful study of the incidence and biological character of these new organisms might provide a clue to the solution of the

* This study was made possible by a grant from the Chemical Foundation, Inc.

¹ Branham (9) in a recent study of the occurrence of anaerobes in normals, influenza, colds, etc., has isolated and studied a number of these organisms.

problem. These organisms, as originally described by Olitsky and Gates, and further studied by Olitsky and McCartney, are anaerobic, Gram-negative bacilli, which pass through Berkefeld V and N filters, and which are culturally and serologically distinct from *B. pneumosintes*.

It has been pointed out in a previous paper (10), that familiarity with the bacterial flora of the upper respiratory tract in health is a prerequisite to correct interpretation of findings in the course of infection. Accordingly, during the first part of the investigation, a group of healthy normals was followed for a period of several months to determine the presence of these filter-passing anaerobes; during the same period search for the organisms was carried out in these subjects when they acquired colds and in many other individuals suffering from typical colds. During the 2nd year, less emphasis was placed upon the study of normals and much time was given to determining the occurrence of these organisms in persons with typical colds. Throughout the work many of the organisms, from both normals and cold cases, were studied culturally and serologically in an endeavor to demonstrate "normal" or "cold" strains.

Methods.

Material.—In the winter of 1925-26, five healthy normal individuals were studied over a period of 5 months for the presence in their nasopharynges of Gram-negative, filter-passing anaerobes of the type noted above. During this period six colds occurred in the group and were similarly observed. At the same time nasal washings from twenty-seven other individuals suffering from typical colds were studied. In the winter of 1926-27, three normals were followed more or less continuously, and material obtained from sixty-seven cold cases for study; fifty-five of these colds occurred in members of the office staff of the American Telephone and Telegraph Company,² and the remainder in hospital personnel, students and patients.

Time of Cultures.—In the normals studied, cultures were made weekly at first and later at 2 to 3 week intervals. In cold cases, cultures were single ones, taken within the first 24 hours of infection.

Method of Obtaining Culture Material.—The material for culture consisted of washings obtained by running various solutions (warmed) gently into the nose

² Permission to make use of the abundant clinical material in this large group of individuals was very kindly granted us by Dr. Cassius Watson of the American Telephone and Telegraph Company.

(5 cc. per nostril) and thence out through the mouth: in addition, subjects usually added 10 to 15 cc. by gargling. During the 1st year of the work, stock buffered broth (pH 7.6) was used for the washings. When the study was extended to the American Telephone and Telegraph Company group, Ringer's solution, as recommended by Olitsky and McCartney (3), was substituted because of the disagreeable taste of the broth. Our results with this fluid were so rarely positive that after a few weeks we returned to the use of broth. Both Ringer's solution and physiological salt solution seem to be bactericidal for these delicate organisms, and successful culture is much more likely when broth is used in washing out the nasopharynx as survival during the period of filtration and transfer is enhanced.

TABLE I.

Incidence of Gram-Negative Anaerobic Organisms in a Normal Subject (F. M. H.) on Seven Successive Cultures.

No.	Date	Culture media				
		Blood plate	Smith-Noguchi	<i>B. coli</i> broth	Dextrose broth and kidney	Dextrose broth and kidney, aerobic or blood broth
1	11-11-25	—	+	—	—	—
2	11-25-25	+	—	—	—	—
3	12- 7-25	—	—	+	—	—
4	12-21-25	—	—	+	—	—
5	1- 4-26	—	—	—	—	—
6	1-18-26	—	—	+	+	+
7	2- 2-26	+	—	—	+	+

+ = growth.

Cultural Methods.—The nasal washings thus obtained were collected in sterile bottles and thoroughly shaken with glass beads. Some of this material was inoculated upon fresh 5 per cent rabbit's blood agar plates and incubated aerobically.

The remainder of the material was passed through Berkefeld V filters. About 0.5 cc. of the filtrate was seeded routinely in various media, as follows: 1925-26, Smith-Noguchi medium, as modified by Gates and Olitsky (11), *B. coli* broth (12), *fresh*, moist, 5 per cent rabbit's blood agar plates and dextrose broth; 1926-27, Smith-Noguchi media. *fresh* rabbit's blood agar plates and rabbit's blood broth. Cultures were incubated anaerobically, anaerobiosis being obtained with the Brown modification (13) of the McIntosh and Fildes (14) jar. Plates were examined after 1 week. Fluid cultures were plated after 1 week, in the case of Smith-Noguchi media plating was repeated after 2 weeks. Study of each case, therefore, took at least 2 to 3 weeks. The reason for the multi-

plicity of medium inoculations noted above lay in the fact that the growth of these delicate organisms is so uncertain that dependence upon one medium alone leads to many negative results although organisms are actually present in the washings. The following record of seven successive cultures in one of the normals studied in 1925-26 illustrates this point, Table I. It will be seen from this table that the incidence of positive single cultures in the complete series is eleven out of thirty-five (31 per cent); actually the washings were positive in six of the seven attempts to recover the organisms, a real percentage incidence of 86 per cent.

During the 2nd year, as noted above, three culture media per washing were deemed sufficient; in our 1st year's experience we had found that positive results were more frequently obtained with the use of blood plates, blood broth and Smith-Noguchi media. An additional reason for our use of the last named culture medium lay in the fact that we were particularly interested in the possible incidence of *B. pneumosintes*, and it has been emphasized by Olitsky and Gates (12), and Gates and Owens (15) that primary cultures of this organism are obtained on this medium only.

Additional cultural methods that were used in an effort to grow the organisms more easily, more surely or in profusion, and which were discarded as unsatisfactory or inferior included inoculation of potato broth, blood agar, pH 7.2, plain broth, sugar broth and blood agar to which filtered fresh tomato juice had been added, agar blebs in rats and chick embryos (16).

For determining sugar fermentations, chocolate broth with 1 per cent of various sugars tested was used. Preparation was as follows: NaCl meat infusion broth, pH 7.2, plus 4 per cent rabbit's blood was heated to 90°C., filtered through paper, and after addition of sugar (1 per cent) was passed through Berkefeld V filters and tubed. After inoculation, tubes were incubated 1 week and any changes in pH determined colorimetrically. The broth in each case was plated and reincubated for check upon growth or survival of the organisms.

Motility.—This was determined by dark-field illumination. There was marked Brownian movement in all cultures tested. Organisms were considered to be motile only when definite progressive motion was observed.

Serological Methods.—Agglutinating sera were prepared by injecting rabbits with live cultures. Three successive daily injections per week for 6 weeks were planned. The marked difficulty, however, of obtaining uniform quantities of the organisms at the times needed led to occasional departures from this routine. Agglutination reactions were done in the water bath at 56°C. (2 hours); readings were made after leaving the tubes in the ice box overnight. Dilutions were carried up to 1:320 whenever sufficient organisms were available. Absorption experiments were not undertaken because of the difficulty in growing sufficient organisms; moreover, cross-agglutination was so infrequent that use of this method was rarely indicated.

Incidence of Gram-Negative Anaerobes.

Incidence in Normals.—A summary of the results of attempts to isolate Gram-negative filter-passing anaerobes from normals in 1925–26 is shown in Table II. During this period broth only was used for nasal washings. The percentage incidence for the group is 78.6 per cent. Case 1 shows a low incidence, 50 per cent. The organisms were recovered from the other individuals at nearly every examination.

TABLE II.

Incidence of Gram-Negative Anaerobic Organisms in Normals, Winter of 1925–26.

Subjects	No. of examinations	Positive cultures	Incidence
			<i>per cent</i>
K. C. M.	14	7	50
F. M. H.	7	6	86
R. F. L.	3	3	100
E. R.	3	3	100
G. S. S.	15	14	94
Totals	42	33	78.6

TABLE III.

Incidence of Gram-Negative Anaerobic Organisms in Normals, Winter of 1926–27.

Subjects	No. of examinations	Positive cultures	Incidence
			<i>per cent</i>
K. C. M.	7	6	85.7
S.	2	1	50
G. S. S.	6	4	66.6
Miscellaneous	5	3	60
Totals	20	14	70

In 1926–27 washings were first made with Ringer's solution, and during this period (about 2 months) the percentage incidence was only 21.4 per cent. We therefore discarded this method and returned to the use of broth. This change proved to be justified as we now obtained a 70 per cent incidence, a result comparable to the findings of the 1st year, Table III.

The net incidence therefore of these organisms, in normals, without

consideration of their type or of pathogenicity, is quite high. When one remembers the extreme difficulty of their cultivation it seems fair to assume that these organisms constitute part of the normal flora of the nasopharynx. That their habitat is the nasopharynx was shown by our ability to recover the organisms regularly from gargled broth and our failure to cultivate them from teeth and gums.

Incidence in Colds.—In 1925-26 six colds occurred in the normal group; Gram-negative filter-passing anaerobes were recovered from half of these. During the year, twenty-seven other colds were cultured with thirteen positives. The percentage incidence for the year was 48.5 per cent. Broth was used for all washings.

TABLE IV.

Comparison between Incidence of Gram-Negative Anaerobic Organisms in Normals and in Colds, 1925-27.

Year	Normals			Colds		
	No. of examinations	Positive cultures	Incidence	No. of examinations	Positive cultures	Incidence
			<i>per cent</i>			<i>per cent</i>
1925-26	42	33	78.6	33	16	48.5
1926-27	20	14	70	9	4	44.4
Totals.....	62	47	75.8	42	20	47.4

During 1926-27 sixty-seven colds were studied. Ringer's solution was used for washings in the first twenty-seven with only one positive result. We therefore went back to the use of broth as in the work with normals. Following this change, local colds showed the organisms again in about the percentage noted for the previous year, 44.4 per cent. In the American Telephone and Telegraph Company group however, the incidence remained quite low (9.4 per cent). Our failure to recover the organisms from these cases may well be attributed to the fact that cultures were transported over considerable distances and that the time elapsing between making the washings and filtration and cultural procedure often ran to 2 or 3 hours.

Table IV shows a summary of the findings in colds and a comparison between these results and those obtained in normals.

In normals, the percentage incidence was based upon several cultures per individual, while one culture per person was the rule in colds. That this was not a source of error in comparing the two groups is shown by the fact that the percentage incidence in normals, obtained by using the results of their first cultures only, was 80 per cent in the 1st year and 75 per cent in the 2nd.

Cultures in which Ringer's solution was used for washings have been purposely omitted as have the American Telephone and Telegraph Company colds, because conditions here were not comparable. If one leaves out of consideration possible variations in strain, and notes only changes in gross incidence, it will be seen at once that Gram-negative anaerobes of the type under consideration appear much less often in colds than under normal conditions. As will appear shortly, we have been able to produce no evidence in favor of the fact that such types as "cold strains" exist. Hence we are justified in concluding that there is a definite reduction in the incidence of these anaerobes in the course of colds, a thing hardly to be expected if these organisms bear an etiological relationship to colds. It is worthy of note that we have demonstrated in a previous paper (10) a similar quantitative reduction, during colds, of most of the organisms that are commonly considered to be normal non-pathogenic inhabitants of the nose and throat, *i.e.*, *Staphylococcus albus*, diphtheroids, Gram-negative cocci, etc.

Attention should be called to the fact that at no time in our work with normals and with colds (we had no cases of influenza) did we recover typical *B. pneumosintes*. We made every effort to cultivate the organisms except the use of animal transmission, and followed carefully all the dicta of Gates and Olitsky (12) and Gates and Owens (15). Also none of our Gram-negative anaerobes agglutinated with the one *B. pneumosintes* serum we were able to prepare.³ The importance of this negative observation lies in the fact that Olitsky and Gates have stated that this organism is never found in normal throats and appears only in influenza.

³ Culture of *B. pneumosintes* (C17, isolated April 10, 1918) kindly given to us by Dr. Olitsky of The Rockefeller Institute, in July, 1925.

Cultural and Biological Characteristics.

Material for Study.—During the course of the investigation, Gram-negative anaerobic bacilli were recovered seventy-six times. From these, twenty-nine strains were preserved for detailed study; of this group eighteen were from normals and eleven from colds. Most of the collected strains were obtained during the 2nd year of the work. The strain of *B. pncumosintes* referred to above was added to the group for parallel study. All but three of the strains were obtained by routine inoculation of the media enumerated above with filtrates from washings. In the case of the exceptions, unfiltered washings were injected into agar blebs in the abdominal walls of rats, and the organisms recovered in two cases from the bleb and in the third from the heart and lungs. These strains were never passed successfully through filters, but morphologically and culturally they were identical with the organisms of the group, therefore they were included for study.

Olitsky and Gates (8) and Olitsky and McCartney (3) have divided the organisms into three definite types (I, II, III) on the basis of morphological, cultural and serological differences. Our results, as will be noted below, although showing rather looser groups, are in general agreement except for our serological findings. In the description that follows we have made use of their classification.

Morphology and Staining.—The organisms are extremely variable in appearance. The chief forms encountered were minute coccobacilli, short fairly straight delicate bacilli (smaller than *B. pfeifferi*), slender curved comma bacilli and long slender spirilliform bacilli. The range in length was from 0.2 to 6.0 micra. Of these, the coccobacilli usually remained true to type (Group II) while the remaining strains (Groups I and III) frequently appeared in one or more of the other forms. In Group I the most distinctive type was the comma bacillus although some of the strains in this group never showed this form. In Group III the most distinctive type was a small delicate straight bacillus. The organisms decolorize easily by Gram's method. Dilute carbol fuchsin was found to be the best counterstain. There was occasional irregular staining in the longer forms, giving a beaded appearance to the organisms.

Motility.—Twelve of the twenty-nine strains showed motile forms. We were unable to demonstrate any relationship between motility and type.

Filtrability.—All strains except the three noted above were recovered from filtrates of washings passed through Berkefeld V candles. Attempts to pass the

organisms through filters after we had them growing failed in all instances but one, and in this case we were unable to confirm the result because of contamination of the culture. It is of special interest to note that in two out of three attempts, passage through chick embryos restored filtrability of the organisms.

Cultural Characteristics.—The organisms are strictly anaerobic. They will survive, however, for many days upon plates or in broth under aerobic conditions.

Growth in Fluid Media.—A certain number of the strains usually grew readily in fluid media (I). Others were characterized by the great difficulty of their cultivation in this medium (II, III). Growth was always diffuse. Of the media used, blood broth gave the most satisfactory results, *B. coli* broth was next and Smith-Noguchi medium least useful. Our strain of *B. pneumosintes* grew very well in these media.

Growth on Solid Media.—Growth on blood agar plates was generally satisfactory, although often scanty and rarely profuse. On several occasions however, we observed very luxuriant growth on some of the plates. We made many attempts to find a cause for this unusual growth, but could not account for it, although we were able to demonstrate that it was not a symbiotic phenomenon. Blood agar plates were much the most satisfactory for preservation of stock strains. Growth on tomato juice blood agar was very good; it was poor upon chocolate agar. That the organisms are not strict hemoglobinophiles was shown by their capacity for moderate growth on plain agar plates. On blood plates there was slight hemolysis at times, most marked in the case of the coccobacillary type.

Colony formation on blood agar plates was very variable, except in the case of the coccobacillary group (II) which grew uniformly as smooth translucent dewdrop colonies, quite similar to *B. pneumosintes* but smaller. A second group (I) appeared often as fine, opaque, conical colonies with smooth edges; they frequently showed variations however, which included granular consistency, rough edges or rounded tops. A third group (III) was entirely inconsistent as to colony formation; its most frequent appearance was as a flat, slightly granular, opaque colony with irregular edges and raised center, other forms included small smooth, large granular rounded, or large smooth brown colonies with metallic sheen, the last often leaving a brown area on scraping the plate. One organism, NSh5, morphologically III, fell into none of these groups; for 2 years it showed fairly consistently flat, granular, translucent colonies with irregular edges. The first and last mentioned types constituted a very small proportion of the whole group studied; accordingly colony formation seems to be an uncertain criterion for classification of the organisms.

Sugar Fermentations.—The paucity of growth of these organisms in fluid media made determination of their sugar fermentations difficult; absence of sugar splitting may occasionally have been due to scanty growth. Fairly definite conclusions have been arrived at by repeated experiments.

Sugars used were dextrose, saccharose, galactose and levulose. Of the twenty-nine strains studied, eight fermented dextrose; the usual final pH was 5.2; these

TABLE V.
Summary of Agglutination Reactions with Six Representative Sera and One B. pneumosintes Serum.

Strains tested	Agglutinating sera						<i>B. pneumosintes</i>
	Type I			Type II	Type III		
	NMc	CH	CLA1	NDs	NSh5	CW	
Group I	NM4.....	-----	-----	-----	-----	-----	-----
	NM13.....	CCCCC3*	-----	-----	-----	-----	-----
	NM15s.....	CCCCC--	-----	-----	-----	-----	-----
	NMc.....	CC2-----	-----	-----	-----	-----	-----
	CE3.....	CCCCC3·	-----	-----	-----	-----	-----
	CH.....	-----	-----	-----	-----	-----	-----
Group II	CLA1.....	CCCC21	-----	-----	-----	-----	-----
	CLB.....	-----	CCCCC--	-----	-----	-----	-----
	CMx.....	-----	-----	-----	-----	-----	-----
	NDs.....	-----	-----	22--	-----	-----	-----
	NMR.....	-----	-----	CCC	-----	-----	-----
	NSS.....	-----	-----	-----	-----	-----	-----
Group III	NM9.....	-----	-----	-----	-----	-----	-----
	CM2b.....	-----	-----	-----	-----	-----	-----
	NDL.....	-----	-----	-----	-----	-----	-----
	NM1.....	-----	-----	-----	-----	-----	-----
	NM2.....	-----	-----	-----	-----	CCC2--	-----
	NM10.....	-----	-----	-----	-----	-----	-----
	NM151.....	-----	-----	-----	-----	-----	-----
	NS1.....	-----	-----	-----	-----	-----	-----
	NSh1.....	-----	-----	-----	-----	-----	-----

Group III	NSh5	---	---	---	CCCCC3	---	---	---
	NSh9	---	---	---	---	---	C22	---
	NSh12	---	---	---	---	---	---	---
	CM1	---	---	---	---	---	---	---
	CM2a	---	---	---	---	---	---	---
	CO	---	---	---	---	---	---	---
	CSh	---	---	---	---	---	CCCCC3	---
	CW	---	---	---	---	---	---	CCCCC2
<i>B. pneumosintes</i>								

* Agglutination dilutions above; 1/10, 1/20, 1/40, 1/80, 1/160, 1/320.

C = complete agglutination, supernatant fluid clear; 3 = same, fluid not quite clear; 2 = gross granular appearance without settling; 1 = fine granular appearance; - = no agglutination.

strains also fermented the other three sugars. *B. pneumosintes* which was tested also split all four sugars. The remaining twenty-one strains failed to ferment any of the sugars tested. The first named eight strains fell into Group I.

Serological Characteristics.—Preparation of the agglutinating sera was beset with many difficulties as noted above. Six sera in all were made; three of these with Group I organisms (two cold strains and one normal), one with a Group II strain (normal) and two with Group III organisms (one cold and one normal strain). A *B. pneumosintes* serum was prepared for parallel tests. We were unable to determine the titre of the Group II serum as we failed consistently to cultivate enough of the homologous organisms to perform satisfactory agglutination reactions.

Table V shows the results. Organisms are arranged according to their morphological and cultural classification. Strains obtained from normals are indicated by the strain symbol N and those from colds by a C. In Group I there were four strains from normals and five from colds, in Group II, four from normals and one from a cold, and in Group III, ten from normals and five from colds.

Reference to the findings in the table shows that there is very little evidence of serological homogeneity in "cold" or "normal" strains or in the three cultural groups. No cross-agglutination occurred between "cold" strains either in or out of the cultural groups. Twice only there was agglutination of "normal" strains by "normal" sera; NM4 and 13 (from same person) by serum NMc (Group I) and NMR by serum NDs (Group II). Twice "normal" strains were agglutinated by "cold" strains: NM15s by serum CH (Group I) and NM2 and NSh9 by serum CW (Group III). Agglutination in Group II was most unsatisfactory for the reasons referred to above; as most of these organisms came from normals and as our principle endeavor was to demonstrate "cold strains," this lack is of little importance. Except for the occasional cross-agglutination noted above we were unable to confirm the findings of Olitsky and McCartney that strains in the cultural groups are serologically related. There was no agglutination of "normal" or "cold" strains by the *B. pneumosintes* serum.

Summary.—Our observations regarding this group of organisms with respect to their morphological and cultural characteristics and classification are in general accord with those of Olitsky and Gates and Olitsky and McCartney, although we have noted rather wide variations in Groups I and III. Contrary to the findings of these authors our strains could not be grouped serologically. Although we have been able to divide the organisms into three classes we have been impressed by the heterogeneous character of the group.

DISCUSSION.

Three outstanding facts appear from this study. (1) The organisms in question are a heterogeneous group, (2) they are nearly always present in the upper respiratory tract and (3) there is a decrease in their incidence during colds.

The lack of homogeneity among the strains was striking. Variations in morphological and cultural characteristics were the rule, even in the subgroups, and very little serological interrelationship was evident. We were unable to demonstrate either "cold" or "normal" strains. In regard to their heterogeneity these organisms resemble such poorly differentiated non-pathogens as *B. coli* or perhaps *B. pfeifferi*. It is true that virulent strains are sometimes noted among such organisms and it may be possible that such pathogenic strains exist among these Gram-negative filter-passing anaerobes, but we were able to produce no evidence in support of this.

The organisms seem to be part of the harmless normal flora of the upper respiratory tract. Probably their percentage incidence would rise higher than that noted by us (75 per cent) if cultural methods could be further improved. The reduction in their incidence in colds makes improbable the assumption that they bear a causal relationship to these infections. In fact, as pointed out above, this decrease is much more in keeping with the conclusion that they constitute part of the normal non-pathogenic flora of the upper respiratory tract.

CONCLUSIONS.

1. A study of the Gram-negative, filter-passing, anaerobic organisms, described by Olitsky and Gates, and Gates and McCartney, has been undertaken with a view to determining their general character and their possible rôle in the causation of the common cold.

2. These organisms seem to constitute part of the normal flora of the upper respiratory tract and would seem to bear no etiological relationship to the common cold.

We wish to acknowledge our indebtedness to Dr. Cassius H. Watson of the American Telephone and Telegraph Company and his professional staff for assistance in obtaining nasal washings from colds.

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STUDIES ON A PARATYPHOID INFECTION IN GUINEA PIGS.

IV. THE COURSE OF A SECOND TYPE OF SALMONELLA INFECTION NATURALLY APPEARING IN THE ENDEMIC STAGE.

By JOHN B. NELSON, PH.D.

*(From the Department of Animal Pathology of The Rockefeller Institute for Medical
Research, Princeton, N. J.)*

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The appearance of a second type of *Salmonella* in a guinea pig population during the endemic stage of a spontaneous outbreak of paratyphoid disease was reported in an earlier paper (1). The earliest cases of the first infection occurred in the summer of 1924. The associated organism became rapidly disseminated throughout the population; particularly through the breeding stock, and for a period of 8 weeks a moderately high specific death rate ensued. The epidemic subsequently declined to an endemic level which has persisted up to the present. The course of the infection was followed by a bacteriological examination of all animals that died in the population under observation.

During July and August, 1926, several strains of a second type of *Salmonella* were isolated from fatal cases. Subsequently there was a slow but general spread of the second organism. These natural occurrences, among the guinea pigs, afforded an opportunity for studying the course of a second infection in an animal population which had been exposed for a considerable period of time to a related but serologically distinct type of organism.

A somewhat similar condition in a mouse population was described by Lynch (2). A spontaneous outbreak of paratyphoid infection, of 2 years duration, was followed by a second outbreak caused by a serologically different type of *Salmonella*. The two types of organism were designated Mouse Typhoid I and II. Some 18 months prior to the second outbreak half of the population was vaccinated with a strain of Mouse Typhoid I. The specific death rate for the

ensuing 7 months was low. There followed a period of fluctuation, with the death rate in general on a higher level, which terminated in a sharp rise. At this time the second type of organism was isolated.

Later, Pritchett (3) encountered a reverse replacement during the course of an artificially induced mouse epidemic. This was started by placing normal individuals in contact with mice which had received *per os* injection of Mouse Typhoid II. After an interval of several months the original Mouse Typhoid II strain was almost entirely replaced by Mouse Typhoid I. The latter type was known to be mildly endemic in the breeding stock and was thought to have been introduced into the particular population by the chance addition of fecal carriers.

The recovery of a second type of *Salmonella* from mice under similar circumstances was reported by Topley (4). An epidemic was started by feeding broth cultures of *Bacillus Gaertner*. Some of the mice which died during the early stage of the epidemic yielded pure cultures of a different *Salmonella* type. The organism showed a close serological relationship to *Bacillus suispestifer* (mutton). In a later stage of the epidemic both types were frequently isolated from the same animal. It should be added that the earlier type corresponded with Mouse and Guinea Pig Type I, and the second organism with Type II.

It may be pertinent to refer, in passing, to the relationship of the mouse and guinea pig types of organism and to their position within the *Salmonella* group. It was previously shown that Mouse Typhoid I and Guinea Pig Paratyphoid I of our epidemic were intimately related if not identical strains of the same *Salmonella* type (5). A similar relationship was established for Mouse Typhoid II and Guinea Pig Paratyphoid II. That the latter are an *aertrycke* form of *Bacillus paratyphi* likewise appears established (1). The relationship of the former, the initial strain, to a distinct *Salmonella* type is less definitely indicated. Amoss and Haselbauer (6) have related Mouse Typhoid I to *Bacillus enteritidis*. For, of three *Bacillus enteritidis* strains, the first was agglutinated to the titer limit by a Mouse Typhoid I antiserum, the second to less than 50 per cent and the third not at all. Upon absorption the first strain reduced the agglutinin content 50 per cent, the second 60 per cent and the third anomalously 50 per cent. A similar relationship, on the basis of direct and reciprocal absorption tests with specific agglutinating serums, was indicated by Sakai (7). The absorption tests, however, revealed a considerable variation in the absorptive capacity of the strains employed. Nelson and Smith (5) reported only a remote relationship between the two rodent strains and two strains of *Bacillus enteritidis*. A specific antiserum which agglutinated the former in a dilution of 1:51,200 agglutinated the latter in a dilution of 1:800 and 1:400 respectively. Absorption tests were not run. There appears to be nearly as wide a variation between different strains of *Bacillus enteritidis* and between the same and the Type I rodent strains as there is between the latter and the Type II rodent strains.

A general discussion of the rodent forms of the paratyphoid-enteritidis group is given by Jordan (8). He divided them into two main types, one related to *Bacillus enteritidis*, the other to an *aertrycke* form of human *Bacillus paratyphosus*.

In the several papers of the present series the two organisms under discussion have been consistently designated as *Bacillus paratyphi* of a given type, I or II. This broad designation has been adopted as a matter of convenience pending more satisfactory evidence for the species relationship of the initial form.

The course of the Type II infection is considered first with reference to its percentage mortality within the population at large and secondly with reference to its cage to cage spread among the breeding stock. The former is computed from the specific deaths occurring within the total estimated population by months. The figures are only approxi-

TABLE I.

Population, Total Deaths, Deaths from Paratyphoid and Percentage Mortality from Paratyphoid, July, 1925, through June, 1926.

Month	Population	Total deaths	Deaths from paratyphoid	Mortality from paratyphoid
				<i>per cent</i>
July.....	401	51	10	2.49
August.....	460	8	5	1.08
September.....	438	27	16	3.65
October.....	500	7	5	1.00
November.....	540	9	3	0.55
December.....	581	15	0	0.00
January.....	470	22	1	0.21
February.....	455	31	11	2.41
March.....	493	24	1	0.20
April.....	491	17	2	0.40
May.....	502	18	0	0.00
June.....	478	33	7	1.46

mations inasmuch as the general population is not stable because of the removal of individuals for experimental purposes. Moreover, it is difficult to classify specific deaths as active cases or carriers which died from some other cause. This applies particularly to the unweaned guinea pigs among which gross lesions may not be apparent. Hence, the specific deaths undoubtedly include a few cases which should be classed as carriers. It is believed, however, that the percentage mortality indicates roughly the progress of the infection. The population by months, the total deaths, the specific deaths and the per-

centage mortality for the period between July, 1925, and July, 1926, are given in Table I.

The data pertaining to the combined infections for the year beginning with July, 1926, when the second type first appeared, are given in Table II.

The percentage mortality for the two periods is presented in graphic form in Fig. 1.

The percentage mortality for the Type I infection displayed a considerable monthly fluctuation during the 2 endemic years beginning

TABLE II.

Population, Total Deaths, Deaths from Paratyphoid and Percentage Mortality from Paratyphoid, July, 1926, through June, 1927.

Month	Population	Total deaths	Deaths from Paratyphoid Type I	Mortality Type I	Deaths from Paratyphoid Type II	Mortality Type II
				<i>per cent</i>		<i>per cent</i>
July.....	571	14	7	1.22	1	0.17
August.....	468	48	30	6.41	3	0.64
September.....	382	32	17	4.45	3	0.78
October.....	463	11	0	0.00	5	1.07
November.....	432	13	2	0.46	5	1.15
December.....	417	64	4	0.95	22	5.27
January.....	436	40	3	0.68	13	2.98
February.....	424	35	1	0.23	11	2.59
March.....	406	49	1	0.24	24	5.91
April.....	454	57	5	1.10	18	3.96
May.....	407	42	1	0.24	11	2.70
June.....	449	35	0	0.00	5	1.11

July, 1925, and July, 1926, respectively. From the curve it may be seen that the highest rates, with a single exception, occurred during warm weather, namely: in July and September of 1925 and in February, June and Augusts of 1926. In general the infection showed a wave-like progress throughout the entire endemic stage. After the high rate of August, 1926, the percentage mortality declined and remained on a low level thereafter. The Type II infection appeared just prior to this peak rate of the initial type, in July, 1926. A relatively quiescent period of 5 months duration followed. During this time there was a slight but steady increase in the percentage mortality. The

period terminated in a sharp rise during December, 1926. There was a decline the 2 succeeding months with a second rise to a slightly higher level in March, 1927. Both of the peaks, it will be noticed, occurred during cold weather. The percentage mortality again declined and continued to fall through June at which time accurate observations were discontinued.

The breeding stock was the only group within the population which was sufficiently stable for a study of the cage spread of the Type II infection. The breeders are segregated in cages containing either four

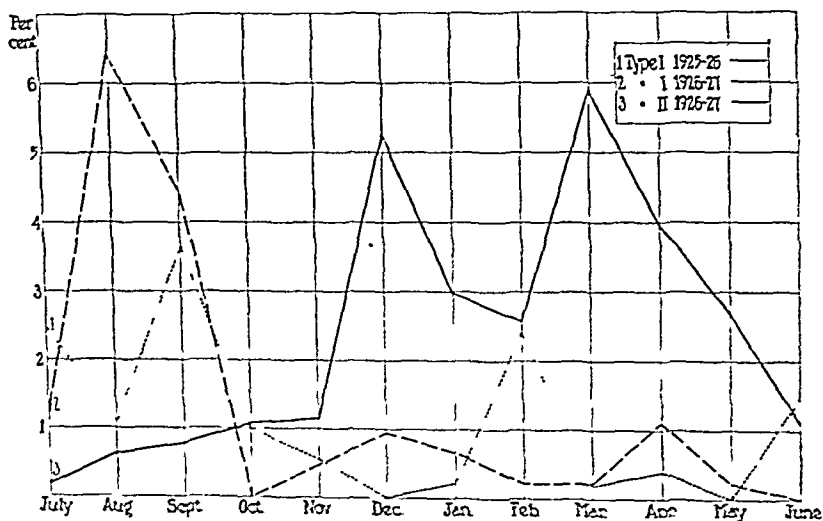


FIG 1. Percentage mortality from Type I infection July, 1925, to June, 1927, and from Type II infection July, 1926, to June, 1927.

or five sows and one boar. Since the appearance of the second form of paratyphoid the sows have been removed to individual isolation units shortly before parturition. The young are born here and are left with their dams for a period of about 2 weeks when they are weaned. At this time the young are transferred to the stock, for general laboratory use, and the sows are returned to their original breeding cages. Until the suckling period is over the majority of young guinea pigs are subjected to direct contact only with their dams or within the litter. Occasionally a premature birth occurring in the

TABLE III.

Spread of Type II Infection in the Breeding Cages July, 1926, to June, 1927.

1.	4.	7. Dec. 26 C.	10. Oct. 26 D.	13. Aug. 26 C.	16.
2. Dec. 26 C. May 27 C.	5. Dec. 26 D. Dec. 26 C. Mar. 27 C. Apr. 27 C.	8. Dec. 26 D. Feb. 27 D. Mar. 27 C.	11.	14. Dec. 26 D. Dec. 26 C. Mar. 27 C.	17.
3. Jan. 27 D.	6. Jan. 27 C. Apr. 27 C.	9. July 26 D. Dec. 26 C. Jan. 27 D. Feb. 27 C. Mar. 27 C. Apr. 27 D. Apr. 27 D. June 27 D.	12.	15. May 27 C.	18. Dec. 26 C.
19. Oct. 26 D. Feb. 27 D. Mar. 27 C.	25. Oct. 26 C. Apr. 27 D. Apr. 27 C.	28. May 27 C. May 27 D.	32. Nov. 26 C.	35. Mar. 27 C.	39. Oct. 26 C. Nov. 26 C. Dec. 26 C. Apr. 27 D.
20. Sept. 26 C. Jan. 27 D.	26. Dec. 26 D. Mar. 27 C. Apr. 27 D.	29.	33.	37. Feb. 27 C. Mar. 27 D.	40. Aug. 26 C. Apr. 27 D.
	27. Oct. 26 D. Feb. 27 D. Mar. 27 D. May 27 C.	31. Nov. 26 C. Dec. 26 C. Jan. 27 C. Apr. 27 C.	34. Jan. 27 C.	38. Feb. 27 C.	41. May 27 C.
42. Sept. 26 C.	44. June 27 C.	49. Dec. 26 C. Apr. 27 D.	48. Feb. 27 D.	51.	54.
43.	46.	52.	45.	50.	53.
		47. May 27 D.			

C., carrier; D., fatal case.

breeding cage brings them in contact with adult animals other than their dam.

Fatal cases of paratyphoid among the unweaned guinea pigs may be attributed in most cases to transfer of the organism from the dam. The transfer may be direct during intra-uterine life or indirect through the ingestion of contaminated feces or milk. Frequently the sow presents a normal appearance. These cases have been common with both types of infection. Formerly the sows were killed and autopsied. Throughout the second infection, however, most of them have been returned to the breeding cages and kept under observation. All of them were designated carriers. In addition, fatal cases have occurred from time to time among the sows, either in the breeding cages or in the isolation units. A cage record of the so called carriers and fatal cases has been kept since the onset of the second infection. A presentation of the data is given in Table III. Each square represents a cage in the order of its arrangement in the breeding room. The notations refer to individual sows. There are three sections of cages, as indicated.

Within a year after the first death from the second infection either fatal cases or carriers were detected in 32 out of 48 cages. The inmates of the remaining cages may have escaped infection entirely. It is more probable, however, that the repeated fecal examination of individuals would have revealed a small proportion of carriers. It is unlikely that there was any carriage of the infection from cage to cage except through the agency of the handlers.

The cages are of metal with solid tops and bottoms and are placed in rows on iron pipe racks. The rows are separated from each other by a wide air space. The cage arrangement precludes any possibility of direct leakage. In some instances infection may have been introduced through the addition of young sows to replace older ones removed by death or because of old age. These sows are drawn from the general stock which always contains a small number of carriers. With the exception of Cage 9, however, replacements have not been added to those cages with a high incidence of infection. It may be said that the breeding stock is cared for by two handlers. The feeding and cleaning in cages from 1 through 47 is always done by the same helper. Similarly, Cages 48 through 54 and in addition the isolation units are attended to by one man.

The spread of infection from cage to cage and within individual cages was gradual. The irregularity which is apparent with the cage

spread may be associated with the nature of the particular group. The breeding stock is composed largely of sows which normally breed and bear young four times a year. During pregnancy it is to be expected that the sow becomes more susceptible to bacterial invasion than during the postparturient periods. The ingestion of a normally tolerated dose of culture during the pregnant state might result in active disease. Or, in the absence of actual disease the lodgment of organisms in the uterus might result in a carrier state with subsequent infection of the young. Again, the ingestion of a sublethal dose of culture during a resting period with localization of the organism in the spleen, liver or intestinal tract might be followed by active disease during a subsequent pregnancy. It is suggested that the continual shifting of the sows together with periodic changes in the susceptibility of individuals were important factors in determining the cage spread of the organism.

In this connection it may be noted that no boars succumbed throughout the course of the second infection. It is to be expected that the number of fatal cases would be less than among the sows since the latter outnumber them five to one. The chances of the boars acquiring infection in the breeding cages are, however, somewhat greater since they remain there continuously except for a short interval during the summer. The sows, on the other hand, are periodically removed to isolation units while the young are suckling. It may be that the more vigorous nature of the boar is accompanied by a more active native resistance. It is believed, rather, that the weakening effect of successive pregnancies on the sow is accountable for the seeming difference in susceptibility. Among younger animals which have not been bred the incidence of fatal cases has shown no consistent difference.

DISCUSSION.

Two years after the initial outbreak of paratyphoid the second infection appeared in the population. Following a quiescent period of 5 months the mortality from the latter type increased abruptly, declined slightly and again increased with a second decline. Both of these waves occurred during cold weather, the first in December, the second in March. Throughout this period the mortality resulting from the initial type was consistently low. The course of the second

infection was somewhat different from that of the initial one. The onset of the latter was abrupt, while that of the former was gradual. The mortality was greater with the initial disease and there was a corresponding increase in the number of fatal cases among adult animals. Apparently the population after long continued exposure to the first infection was better able to resist the second.

It seems clear, however, that the factors instrumental in holding the Type I infection to a low level were not equally active in combating the second type. A number of possible factors were discussed in a preceding paper (9). These will be reconsidered from the standpoint of their bearing on the spread of the second infection. It may be said that prior to the initial outbreak the guinea pig population had been free from specific disease, of epidemic proportions, for a period of 6 years. It was suggested that during this period there was an accumulation of individuals of low resistance. The natural removal of these animals during the early part of the epidemic left a stock better able to resist invasion and among which the infection ultimately declined to a low level. It may be supposed that natural selection continued to operate throughout the endemic stage tending to maintain a stock of normal resistance. It might be argued, however, that the less severe environmental conditions imposed upon the population during this long period would favor the reappearance of animals of low resistance. The unequal course of the two infections is, in part, opposed to such a view. Moreover, the reappearance of a weak strain of guinea pig should tend to increase the number of Type I cases. Actually these declined. It is suggested that the difference in the onset of the two infections may be attributed to the more uniform nature of the population, as regards natural resistance, which obtained at the appearance of the second type. The latter enjoyed a long free period which was not observed with the former.

With due regard to the significance of natural resistance and selection in checking the initial infection and delaying the second it is apparent that some additional factor or factors must have been operative. Fluctuation in the killing power and the invasiveness of the parasite are naturally suggested. As previously noted the question of a possible alteration in these characters with the Type I organism was not satisfactorily answered from experimental evidence (9). It was

tentatively suggested, however, that the organism was gradually adjusted to the population on a lowered level of virulence. Likewise, the possibility of similar differences between the second type and the initial one after its long sojourn in the guinea pig population has not been settled. Intraperitoneal injection of graded amounts of the two types into guinea pigs and mice failed to demonstrate any consistent variation. The inaccuracies inherent in the method employed are regarded as too great to warrant any final statement concerning the comparative invasive power of the two types.

Specific immunity was earlier considered as a factor in the decline of the initial infection. Its importance in the population at large was minimized but it was not denied that the resistance of certain groups might have been raised. It is certain that conditions were favorable for immunization within the breeding group during the endemic stage of the earlier infection. The Type I organism was widely disseminated throughout the cages of the unit and the ingestion of small doses of culture by individual sows must have commonly occurred. The postmortem examination of so called carriers, sows whose litters had shown one or more cases of paratyphoid, gave indirect evidence of an acquired immunity. In some instances healed or inactive lesions were found in the abdominal organs. The blood serum frequently agglutinated the homologous organism. It may be said, too, that a small number of sows examined shortly after the appearance of the second infection showed a much higher agglutinin titer against the initial organism than against the second type. There is a suggestion that natural immunization with the earlier form afforded a protection which was largely type-specific and accountable in part for the subsequent dominance of the second infection.

SUMMARY.

The course is considered of a second type of *Salmonella* infection naturally appearing in a guinea pig population during the endemic stage of an earlier outbreak. After a quiescent period of 5 months the percentage mortality increased abruptly; fluctuated, with a second rise during the 9th month; and then declined. With the exception of a high rate during the 2nd month the percentage mortality from the initial infection tended to remain on a low level.

The spread of infection in the cages of the breeding stock is recorded from the time of the first fatal case. There was a slow but general dissemination of the second organism through the group. Fatal cases were confined solely to the sows. It is suggested that a lowered individual resistance occurring during pregnancy might be associated with the irregular cage spread and with the apparent difference in susceptibility of the sexes.

Natural host resistance, virulence of the organism and acquired host resistance are discussed from the standpoint of their bearing on the unequal distribution of deaths from the two infections.

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ETIOLOGY OF OROYA FEVER.

X. COMPARATIVE STUDIES OF DIFFERENT STRAINS OF *BARTONELLA* *BACILLIFORMIS*, WITH SPECIAL REFERENCE TO THE RELATIONSHIP BETWEEN THE CLINICAL TYPES OF CARRION'S DISEASE AND THE VIRULENCE OF THE INFECTING ORGANISM.

BY HIDEYO NOGUCHI, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

PLATES 7 TO 10.

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Dr. Sebastian Lorente, Director of the National Department of Public Health of Peru, during a visit to New York in 1926, expressed the wish that experimental studies of Carrion's disease might be further extended and agreed to forward specimens of patients' blood to The Rockefeller Institute for the purpose. Six samples of blood were received from Dr. Lorente on December 29, 1926, and six more on April 1, 1927. Comparative studies of the strains of *Bartonella bacilliformis* isolated from these specimens form the subject matter of the present report. I wish at the outset to express to Dr. Lorente my appreciation of the cooperation which made the work possible.

Isolation of Bartonella bacilliformis from Specimens of Patients' Blood.

The blood was collected in sterile vacuum bulbs containing dry sodium citrate sufficient to prevent coagulation (0.1 gm. per 10 cc. of blood). The containers were hermetically sealed and shipped in the steamer's refrigerator from Lima to New York, the time required for transportation being about 14 days. Three of the first six samples were unsuitable for cultural study, having become contaminated with extraneous bacteria by leakage of the container during transportation. The second shipment (Cases 7 to 12) arrived in perfect condition. *Bartonella bacilliformis* was obtained in pure culture from all six

specimens of the second lot and from the three uncontaminated samples of the first lot, nine strains in all being isolated.

The blood was inoculated into leptospira medium by the titration method, that is, several dilutions were made of each specimen (1:10, 1:100, 1:1,000, 1:10,000, 1:100,000), and a tube of culture medium was inoculated for each dilution and one for the undiluted citrated blood, a total of six tubes of medium for each sample of blood. The amount of inoculum was 0.2 cc.

Dark-field examination revealed no motile organisms. In stained smears forms resembling *Bartonella* were found, but they were not sufficiently numerous or characteristic to be identified with certainty.

Case 1, F. A., 18 years old, resident of Canta, Department of Lima. Had been ill 3 months. Verruga suspected because of patient's residence and his symptoms. Erythrocytes 2,900,000. Leucocytes 7,000; neutrophils 72 per cent, eosinophils 1 per cent, monocytes 7 per cent, lymphocytes 20 per cent; no basophils. *Plasmodium falciparum* was present in the blood. Temperature 37.8°C. at time blood was taken.

Case 2, J. M., 45 years old, resident of Oroya, Department of Junin. Had been ill 2 months. Generalized miliary verrucous eruption as well as subcutaneous nodules. There had been fever of remittent type, but patient was afebrile at the time of taking blood. Erythrocytes 2,500,000. Leucocytes, 13,600; neutrophils 65 per cent, eosinophils 2 per cent, basophils 1 per cent, monocytes 10 per cent, lymphocytes 22 per cent.

Case 3, G. H., 20 years old, resident of Matucana, Department of Lima. Had been ill 15 days. Verrucous eruption of miliary type, scattered. Fever of remittent type still present, highest temperature 40°C. Erythrocytes 3,400,000. Leucocytes, 9,000; neutrophils 69 per cent, eosinophils 1 per cent, monocytes 8 per cent, lymphocytes 22 per cent; no basophils. Barton's bodies present, bacilliform type. Wassermann reaction positive.

Case 4, L. W., 37 years old, resident of Oroya, Department of Lima. Had been ill 1 month. Verrucous eruption of subcutaneous miliary form, scattered, localized chiefly in lower limbs. Patient had no fever at time blood was taken.

Case 5, A. F., 31 years old, resident of Chosica, Department of Lima. Had been ill 4 months. Miliary eruption and subcutaneous nodules were present on both arms and on thorax. Fever of remittent type still present; highest temperature 39.5°C., temperature at time blood was taken 37.8°C. Erythrocytes 1,920,000. Leucocytes 4,600; neutrophils 40 per cent, eosinophils 3 per cent, basophils 1 per cent, monocytes 9 per cent, lymphocytes 47 per cent. Wassermann reaction positive.

Case 6, F. T., 28 years old, resident of Matucana, Department of Lima. Had been ill 2 months. No eruption at time blood was taken, but for 1 month patient

had subcutaneous miliary lesions. Erythrocytes 2,460,000. Leucocytes, 6,400; neutrophils 73 per cent, eosinophils 2 per cent, monocytes 2 per cent, lymphocytes 23 per cent; no basophils.

Case 7, S. N., 22 years old, resident of Puruhuay (Chosica), Department of Lima. No fever. Generalized verrucuous eruption of miliary and nodular types. Erythrocytes 3,480,000. Leucocytes 4,400; neutrophils 72 per cent, eosinophils 1 per cent, basophils 1 per cent, monocytes 4 per cent, lymphocytes 22 per cent.

Case 8, J. A., 33 years old, resident of Canta, Department of Lima. Had been ill 6 months. No fever. Generalized miliary eruption. Erythrocytes 3,100,000. Leucocytes, 4,000; neutrophils 65 per cent, eosinophils 1 per cent, monocytes 8 per cent, lymphocytes 26 per cent; no basophils.

Case 9, J. M., 19 years old, resident of Chosica, Department of Lima. Had been ill 15 days. Continuous fever, 39°C. No eruption. (Malignant verruga?) Erythrocytes 1,250,000; normoblasts 4 per cent, erythroblasts 2 per cent. Anisocytosis, anisochromia, polychromatophilia. Leucocytes 8,400; neutrophils 73 per cent, eosinophils 2 per cent, monocytes 3 per cent, lymphocytes 22 per cent; no basophils. Barton's bodies present, bacilliform type predominating. Jolly bodies also present.

Case 10, E. C., 39 years old, resident of Chosica, Department of Lima. Had been ill 1 month. No fever. Verrucous eruption, miliary and nodular, scattered. Erythrocytes, 5,080,000. Leucocytes 5,200; neutrophils 44 per cent, eosinophils 7 per cent, basophils 1 per cent, monocytes 7 per cent, lymphocytes 41 per cent. Barton's bodies of coccoid type present.

Case 11, M. C., 27 years old, resident of Chosica, Department of Lima. Had been ill 12 days. No fever, and no eruption at time blood was taken. Erythrocytes 1,080,000; normoblasts 9 per cent, erythroblasts 2 per cent, neutrophilic myelocytes 3 per cent. Megalocytes. Leucocytes 22,200; neutrophils 88 per cent, monocytes 3 per cent, lymphocytes 9 per cent. No eosinophils or basophils. Barton's bodies, coccoid form, present.

Case 12, M. L., 28 years old, resident of Matucana, Department of Lima. Had been ill 1 month. No fever. Eruption of miliary type, discrete. Erythrocytes 4,800,000. Leucocytes 4,900; neutrophils 54 per cent, eosinophils 5 per cent, monocytes 10 per cent, lymphocytes 31 per cent; no basophils. Barton's bodies present, coccoid form predominating.

Comparative Studies of the Strains Isolated.

Appearance of Growth (30°C.).

On leptospira medium there is a light grayish, usually homogeneous, but sometimes finely granular, translucent layer of growth at the top of the column of medium. Single isolated colonies form denser central masses and are surrounded by a hazy zone of outgrowth from the periphery. All the strains pre-

sented a similar appearance except Nos. 9 and 12, which grew faster and more densely than the others. Horse blood agar plates or slants yield minute grayish, raised, shiny, firm miliary colonies which appear transparent but are grayish when covered with a layer of saline solution. They adhere to the medium and are difficult to scrape from the surface. Strains 9 and 12 form somewhat larger colonies, which reach a diameter of 2 mm. within 4 or 5 days.

Motility (Dark-Field Observations).

Cultures on leptospira medium or horse blood agar slants or plates contain very actively motile forms for the first 4 to 6 days, especially in the condensation water of the blood slants, but the organisms invariably become motionless sooner or later. The flagella apparently become abnormally large under the unfavorable conditions existing in a semisolid medium and are thrown off; for detached flagella, so large as to be visible by dark-field examination, appear in cultures about a week old (Figs. 32 and 33).

I have been unable to detect any differences in movement among the strains studied; they all exhibit rotation and progression, singly or in groups of two or three, or in masses. Occasionally there is no motility at all even in young cultures, owing undoubtedly to unfavorable conditions of cultivation.

Staining Properties and Dimensions.

All the strains are frankly Gram-negative and take basic fuchsin rather poorly. Giemsa's solution gives satisfactory results and brings out the fairly sharp contours of the organisms in cultures which have been grown 4 to 6 days on the surface of blood agar slants or plates. This definition of the form is quickly lost as the cultures grow older. Organisms grown on leptospira medium or in the condensation water of a blood agar slant stain only poorly, and it is often impossible to recognize individuals; nevertheless they thrive well and remain viable under these conditions much longer than on the surface of slants or plates. There is a tendency for most of the strains to take up the stain more intensely in the interior portion of the body than on the outer layer. Often the chromatophilic material is seen to be heaped up toward either end, giving the appearance of a diploid coccobacillus. Sometimes the accumulation is in nodular masses at irregular intervals throughout the entire length, the effect simulating a chain of ill stained cocci, while in shorter forms there may be only a single deeply stained dot. The organisms often assume a kind of triangular or elongated wedge shape.

In fresh preparations of young, actively motile cultures, the organisms all appear to be of the same size and shape, but certain variations in size and form are recognizable in stained smears (Plates 7 and 8). Special care has been taken to exclude various external factors which might influence the comparison. For example, cultures have been grown on the same medium (horse blood agar plates prepared on the same day with the same material) for 6 days at 30°C.

under identical conditions, and film preparations have been made in the same way (the culture put into a drop of distilled water, the mixture spread on the slide, and dried in the air for a given period), fixed in methyl alcohol for 5 minutes, and then stained with Giemsa's solution in the same jar for 30 minutes. Another set of slides has been treated by Gram's method and counterstained with dilute carbol fuchsin, washed, blotted, and dried in the air. The measurements given in Table VI, therefore, should be fairly reliable. Strain 7 (Figs. 5 and 6) is distinctly coarser and Strain 11 (Figs. 13 and 14) decidedly finer than the rest. There is, however, a gradual transition, some approaching the coarser type and others the finer, hence striking differences are brought out only by comparing the two extremes. Some strains are short, others more rod-shaped.

Flagella (Zettnow-Fontana Combination Stain).

Determination of the type of flagella in newly isolated pathogenic micro-organisms is not of morphological interest merely; it may prove to be of immunological importance. Orcutt¹ has demonstrated that the serological specificity of certain organisms resides in the flagella, and Bauer² recently found in this laboratory that serological types of tetanus bacilli possess dissimilar flagella; for example, Type III, having a single unipolar heavy spiral flagellum, and Type IV, having peritrichal, rather coarse regularly wavy flagella, of moderate length, are quite distinct from the other types. All strains of *Bartonella bacilliformis* were therefore stained for flagella by a modified procedure, in which Zettnow's mordant is followed by reduction with Fontana's ammoniac silver nitrate. The cultures used were those utilized for the measurements of size, that is, the surface growth on horse blood agar plates after 6 days at 30°C.

Certain strains proved to have comparatively shorter, smoother, and more delicate flagella than others (Plate 9). Strains 3, 9, 10, 11, and 12 (Figs. 22, 26 to 29) are of this type, while Strains 2, 7, 8 (Figs. 21, 24, and 25), and the two old strains, S.A.³ and P.5⁴ (Fig. 30) have distinctly more wavy or spiral and coarse appendages. The number of flagella seems to vary among different strains, some showing a single flagellum, others as many as four. In all instances, however, the flagella are unipolar. Short flagella scarcely exceed 3μ , long ones may measure 10μ or more.

Pathogenicity.

Macacus rhesus monkeys were used to determine the virulence of the original samples of blood, as well as of the newly isolated cultures,

¹ Orcutt, M. L., *J. Exp. Med.*, 1924, xl, 43, 627.

² Bauer, J. H., not yet published.

³ Noguchi, H., and Battistini, T. S., *J. Exp. Med.*, 1926, xliii, 851.

⁴ Noguchi, H., *J. Exp. Med.*, 1927, xlv, 175.

the tests being made by three different methods. (1) The effects of local inoculation of the original samples of blood were simultaneously compared by intradermal inoculation of each specimen into separate sites on the abdominal skin of the same monkey (the experiment was done in duplicate), previous experience having shown⁵ that an experimental local lesion in *Macacus rhesus* remains confined to the site of inoculation, and that several intradermal inoculations with different materials may be made simultaneously on the same animal. (2) The effect of local inoculation of each of the cultures isolated from the blood

TABLE I.

Tests of Infectivity of Blood, Cases 1 to 6. Multiple Intradermal Inoculations, Dec. 29, 1926.

Case No.	<i>M. rhesus</i> 1A	<i>M. rhesus</i> 2A
1	—	—
2	—	—
3	—	—
4	—	—
5	—	—
6	—	—
Blood cultures, Jan. 6, 1927.		
	—	—
Blood cultures, Jan. 24, 1927.		
	+ (1:100)	+ (1:100)

was compared in the same animal with that of the inoculation of passage strains of known virulence. (3) Each strain was individually tested on a monkey by both intradermal and intravenous inoculation.

The first two types of test have an advantage over the third in that the variations in the susceptibility of individual monkeys is not involved. The third method is the more usual one, but in testing a large number of strains it is necessary to economize in monkeys, hence the factor of individual variation may enter into consideration. The results in this series of animals, however, were determined not only by local reactions but also by blood cultures at appropriate intervals.

⁵ Noguchi, H., *J. Exp. Med.*, 1927, xlv, 455.

For virulence tests, cultures grown for 8 days on leptospira medium at 30°C. and those grown for 4 to 6 days on horse blood agar slants and plates at the same temperature were pooled, the mixture comprising first, second, and third generation cultures of a given strain. The original samples of blood were tested again simultaneously with the cultures, a procedure which yielded interesting results, inasmuch as the blood, in contrast to the cultures, produced no cutaneous lesions. The results of the various tests are outlined in the tables.

Table I summarizes the results of the direct pathogenicity tests of the blood of Cases 1 to 6 inclusive. No local lesions were induced,

TABLE II.

Parallel Infectivity Tests of Blood of Cases 2, 3, and 5, and of Cultures Obtained from the Blood. Multiple Intradermal Inoculations, Feb. 7, 1927.

Case No.		<i>M. rhesus</i> 3A
2	Blood.....	—
	Culture.....	++
3	Blood.....	—
	Culture.....	++++
5	Blood.....	—
	Culture.....	++++

though invasion of the blood by *Bartonella bacilliformis* occurred, as shown by the results of blood culture in both animals 26 days after inoculation. The cultures from Cases 1, 4, and 6 were contaminated, those from Cases 2, 3, and 5 yielded marked local lesions (Table II; Fig. 31). Similar results were obtained with the specimens from Cases 7 to 12, the original blood being non-virulent for the skin of the monkey, while the cultures in three instances (Cases 9, 10, 11) gave rise to typical verruga lesions. Parallel tests of the blood and the cultures in Cases 7 to 12 are recorded in Tables III and IV. Strains 7 to 12 were tested separately on different monkeys (Table V) with results which agree with those of the multiple tests.

The experiments show that of nine strains tested, six possessed definite specific pathogenicity for *Macacus rhesus*; the other three

were non-pathogenic, as shown by three separate tests. The three non-virulent strains (7, 8, and 12) came from cases of benign miliary or nodular verruga, in two of which the microscopic examination of the blood had been negative for *Bartonella*; in Case 12 *Bartonella* was present in the blood, though the red count was practically normal. The six virulent strains, with the exception of Strain 10, came from patients whose blood showed either microscopically detectable num-

TABLE III.

Parallel Infectivity Tests of Blood of Cases 7 to 12 and of Cultures Obtained from the Blood. Multiple Intradermal Inoculations, May 3, 1927.

Case No.		<i>M. rhesus</i> 4A	<i>M. rhesus</i> 5A
7	Blood.....	—	—
	Culture.....	—	—
8	Blood.....	—	—
	Culture.....	—	—
9	Blood.....	—	—
	Culture.....	+++	+++
10	Blood.....	—	—
	Culture.....	+++	+++
11	Blood.....	—	—
	Culture.....	++++	++++
12	Blood.....	—	—
	Culture.....	—	—
Control (P. 5 strain, from verruga nodule)		++++	++++

bers of *Bartonella bacilliformis* or marked anemia, or both. In three of these cases there were no skin lesions, and the red counts were very low (1,080,000 to 1,250,000). These represent the pure septicemic form of Carrion's disease, without cutaneous involvement.

The fact that the virulent strains came from the severe types of Carrion's disease, and the non-virulent ones from benign verruga seems to indicate that the highly fatal disease is caused by strains possessing a greater virulence. The first strain of *Bartonella bacilli-*

formis isolated,³ which was obtained from the blood of a fatal case of Carrion's disease, was found to produce in monkeys of average susceptibility the clinical picture of a human case of verruga, but in unusually susceptible animals, which are rather rare, it induced a fatal infection similar to that of human Oroya fever. The strains from the severe

TABLE IV.

Parallel Infectivity Tests of the Original Blood, of Cultures on Leptospira Medium, and of Blood Broth Mixtures, Cases 7 to 12. Multiple Intradermal Inoculations, May 18, 1927.

Case No.	<i>Macacus rhesus</i> 6A			
	Original blood	Cultures		
		Leptospira medium	Blood broth	
			36°	37°
7	—	—	—	—
8	—	—	—	—
9	—	+++	—	—
10	—	++	—	—
11	—	+	—	—
12	—	+	—	—

TABLE V.

Individual Tests of Strains 7 to 12. Intradermal and Intravenous (2 cc.) Inoculations, July 1, 1927.

Strain No.	Monkey No.	Skin lesions	Blood cultures
7	7A	—	— (Aug. 8, 1927)
8	8A	—	—
9	9A	+	+
10	10A	+++	+
11	11A	++++	—
12	12A	—	—

cases of the present series have not so far, in the small number of monkeys inoculated, reproduced the fatal Oroya fever, but they have invariably induced cutaneous lesions similar to those occurring in benign human cases, while the strains isolated from the blood of benign verruga have not proven sufficiently virulent to set up any infection in the same monkeys.

TABLE VI.
Summary of Data.

<i>B. bacilliformis</i> strain No.	Clinical data				Observations on cultures							Pathogenicity for <i>M. rhesus</i>		
	Fever	Erythrocyte count	<i>Bartonella</i> in blood	Eruption	Duration of illness	Cultural titer of blood	Growth on leptospira medium	Growth on blood agar	Measurements				Flagella	
									Predominant forms	Extremes			Character	Length
									Width	Length				
2		2,500,000		General military	2 mos.	1:10,000	30°C. Light gray- ish haze	30°C. Minute, almost transparent, raised, shin- ing, firm, round colonies	0.3 × 0.8μ	0.25-0.35μ	0.5-1μ	Unipolar, 2 or more, smooth	3-5μ	+
3		3,400,000	+	Few eruptions	15 days	1:1,000	"	"	0.3 × 1μ	0.25-0.4μ	0.4-1.2μ	Similar, but spiral	3-5μ	+
5		1,920,000		Military and nodular	4 mos.	1:10,000	"	"						+
7	-	3,480,000		General military and nodular		1:10	"	"	0.4 × 1.4μ	0.3-0.4μ	0.8-1.6μ	Unipolar, 1 or 2; spiral	5-10μ	-
8	-	3,100,000		General military	6 mos.	1:10	"	"	0.3 × 1.2μ	0.25-0.3μ	0.5-1.4μ	Same as 7	5-10μ	-
9	39°	1,250,000	+	-	15 days	1:10,000	" (denser)	" (coarser)	0.3 × 1μ	0.25-0.3μ	0.4-1.4μ	Unipolar, 2 or 3; smooth or wavy	3-6μ	+

10	-	5,050,000	+	Few mili-ary nodular	1 mo.	1:1,000	Light gray- ish haze	Minute, almost transparent, raised, shin- ing, firm, round colonies	0.35 × 1 μ	0.3 -0.4 μ	0.3 -1.2 μ	Unipolar, 1 to 3; smooth or slightly wavy	3-8 μ	+
11	-	1,080,000	+	-	12 days	1:100,000	"	"	0.2 × 1 μ	0.2 -0.25 μ	0.3 -1.2 μ	Same as 10	3-8 μ	+
12	-	1,800,000	+	Few mili-ary	1 mo.	1:100	"	"	0.3 × 0.7 μ	0.3 -0.4 μ	0.5 -1.2 μ	" " 10	3-5 μ	-
S.A.							(denser) Light gray- ish haze	(coarser) Minute, almost transparent, raised, shin- ing, firm, round colonies	0.25 × 1 μ	0.25-0.35 μ	0.45-1 μ	Unipolar, 2 to 4; spiral	5-10 μ	+
P. 5 (nod- ule)							"	"	0.25 × 1 μ	0.2 -0.3 μ	0.3 -1.2 μ	Same as S.A.	5-10 μ	+

As has previously been suggested, the variety of types of human *Bartonella* infection may be accounted for by variations in the susceptibility of different individuals or different tissues of the same individual, or by variations in the virulence of the microorganism, or by both. The present experiments appear to emphasize more particularly the factor of variation in virulence of the parasite. According to clinical observations, the skin lesions more usually follow the acute febrile anemic stage (*fièvre grave de Carrion*, or Oroya fever); the lesions in these instances are evidently caused by a strain virulent enough to have invaded the blood. Yet there are cases in which the infection is chiefly confined to the cutaneous tissues, the anemia being very mild, and strains isolated from such cases would presumably be less virulent, as was found to be true in the present experiments. In instances in which the severe bartonellemia supervenes in the course of an apparently benign verruga, we may conclude that the strain belonged to the virulent type, but that the individual was of the resistant type, and that the defenses of the resistant organism were finally overcome by continued constitutional disturbances, due perhaps to the presence of the parasite in question, perhaps to some other cause. Phenomena of this sort are not uncommon in infections with other organisms which may give rise to septicemia following local infection (streptococcus, staphylococcus, the tubercle bacillus).

Serologic Studies.

Suspensions of *Bartonella bacilliformis* which would be suitable for agglutination tests are difficult to obtain, owing to the fact that the masses of organisms are well nigh impossible to disperse. Complement fixation tests proved easier to interpret.

Two samples of immune sera, obtained by inoculating rabbits intravenously on several occasions at appropriate intervals with live cultures of Strain S. A.³ were employed against a non-fixing dose of saline suspensions of plate cultures (horse blood agar) of each strain of *Bartonella bacilliformis*. The suspensions were heated at 60°C. to kill the microorganisms and the immune sera inactivated at 56°C.

All the strains gave complete fixation with the antiserum with the exception of Strain 12, which gave partial fixation (30 per cent). The

results indicate that on the whole the strains belong to the same serologic group.

DISCUSSION.

Carrion's disease offered a singularly complex problem, because of its two clinically dissimilar aspects, the grave fever of Carrion (Oroya fever), and benign verruga. The presence of the endoglobular bodies discovered by Barton⁶ practically settled the etiology of the severe cases, but the relation of Barton's bodies to the cutaneous lesions of verruga remained obscure, since it had not been proven that the same bodies were present in the blood or skin lesions of patients with the mild cutaneous disease. Moreover, while the cutaneous tissues affected were definitely infective, inducing similar skin lesions in monkeys, the parasites present in such large numbers in the blood of patients with malignant anemia, with or without skin lesions, appeared to be non-infective for monkeys, inducing neither skin lesions nor systemic infection so far as could be determined. It has recently been shown, however, that the apparently negative findings were not due to the absence of *Bartonella bacilliformis*, but to the fact that, except in severe systemic infection, the parasite cannot be detected by microscopic examination, its presence being revealed only by a suitable culture method.⁷ The unsolved portion of the problem of the etiology of Carrion's disease has been cleared up by the cultural procedure, which permits the ready isolation of *Bartonella bacilliformis* and its detection even when it is present in extremely small numbers.

The importance of the selection of the culture method cannot be over-emphasized, since on the special features of the medium used depended the success of the entire investigation. Had blood agar been used for the isolation experiments—and it is by far the best of the many other media tried—many strains would undoubtedly have been missed, some because growth of the microorganism is not uniformly successful on this medium, some because of loss of virulence due to the frequent subculturing required with blood agar cultures. Once the parasite had been obtained in culture on the semisolid leptospira medium, and its special properties studied, it became possible to discover the causes

⁶ Barton, A. L., *Cron. med.*, Lima, 1909, xxvi, 7.

⁷ Noguchi, H., *J. Exp. Med.*, 1926, xlv, 697.

for the apparently conflicting clinical and experimental evidence. Moreover, by special histological technique⁸ it was shown that *Bartonella bacilliformis* is microscopically detectable in large numbers in human and experimental lesions, where it had previously escaped detection.

The reason for the difference in pathogenicity between the endoglobular parasites, which are present in such large numbers in verruga maligna, and those existing in the skin lesions of verruga benigna has not been brought to light. The fact that in the first mentioned condition the microorganism is incapable of inducing in monkeys anything more than a slight transitory blood invasion, detectable only by blood culture, explains the negative results obtained by the Harvard Commission⁹ with blood from severe Oroya fever, results which seemed unmistakably to indicate a totally different etiology for Oroya fever and verruga peruana.

SUMMARY.

Through the cooperation of Dr. Sebastian Lorente, Director of the National Department of Public Health of Peru, nine strains of *Bartonella bacilliformis* have been isolated, by means of the semisolid leptospira medium, from nine of twelve specimens of blood withdrawn from cases of verruga and forwarded from Peru under conditions of refrigeration. The cultural titer of the blood specimens immediately after their arrival (2 weeks after withdrawal) varied from 1:10 to 1:100,000. Blood from the severe anemic type of the disease, in which there was no eruption, had the highest titer. Blood agar slants yielded irregular results, but some strains grew well on these media.

⁸ The technique is that used by Nicholson (*J. Exp. Med.*, 1923, xxxvii, 221) for the detection of *Rickettsia* in tissues. Fixation in Regaud's fluid (*Arch. Anat. Micr.*, 1910, xi, 296), which consists of 4 parts of 3 per cent potassium bichromate and 1 part of commercial formalin, is followed by staining with Giemsa's solution, according to the method recommended by Wolbach (*J. Med. Research*, 1919-20, xli, 1). 1.25 cc. of Giemsa's solution (Grübler's, for bacteria) is diluted with 1.5 cc. of methyl alcohol and 50 cc. of distilled water to which has been added 3 drops of 1 per cent sodium bicarbonate.

⁹ Strong, R. P., Tyzzer, E. E., Sellards, A. W., Brues, C. T., and Gastiaboru, J. C., Report of first expedition to South America, 1913, Harvard School of Tropical Medicine, Cambridge, 1915.

Morphologically the strains differed very little in fresh preparations examined by dark-ground illumination. In stained preparations some strains appeared coarser, others finer than the average. Special staining indicated that the flagella were characteristically unipolar and varied in number from one to four, some strains showing distinctly more wavy and heavier flagella than others. Young cultures grown on the surface of horse blood agar for 3 to 6 days show individuals with fairly sharp contours, short rods, often varying in thickness toward one or both ends, being intermingled with smaller oval or coccoid elements. Some strains show a predominance of bacillary, some of coccobacillary forms. It is not known whether these features are inherent or are due to conditions of growth, which, though identical, may react differently upon different strains. Definiteness in outline disappears with the age of the culture.

More striking variations are found in the virulence of the different strains for the monkey (*Macacus rhesus*). Three of the nine strains isolated proved to be non-pathogenic for the monkeys. All three of these were derived from cases of benign verruga. The remaining six strains all gave rise to local lesions when intradermally inoculated and were recovered in culture from the blood of the animals. So far, severe anemia has not developed in any of the monkeys.

It is significant that most of the severe cases yielded virulent strains, while some of the strains from benign verruga were non-pathogenic. It appears highly probable that the severe form of Carrion's disease is, in general, caused by a virulent strain, while the benign forms are due to a strain of low virulence. On the other hand, a virulent strain may cause benign verruga in unusually resistant persons and a weak strain may give rise to severe blood infection in unduly susceptible individuals. The form of Carrion's disease is probably determined primarily by the inherent virulence of the strain of *Bartonella bacilliformis* and is modified secondarily by individual predisposition in a given case.

An interesting phenomenon brought out by the present investigation was the failure of the nine human blood specimens to induce local verruga in the same monkeys in which the corresponding cultures, inoculated simultaneously at separate sites, gave rise to typical lesions. Yet the original blood samples were shown by cultivation to have

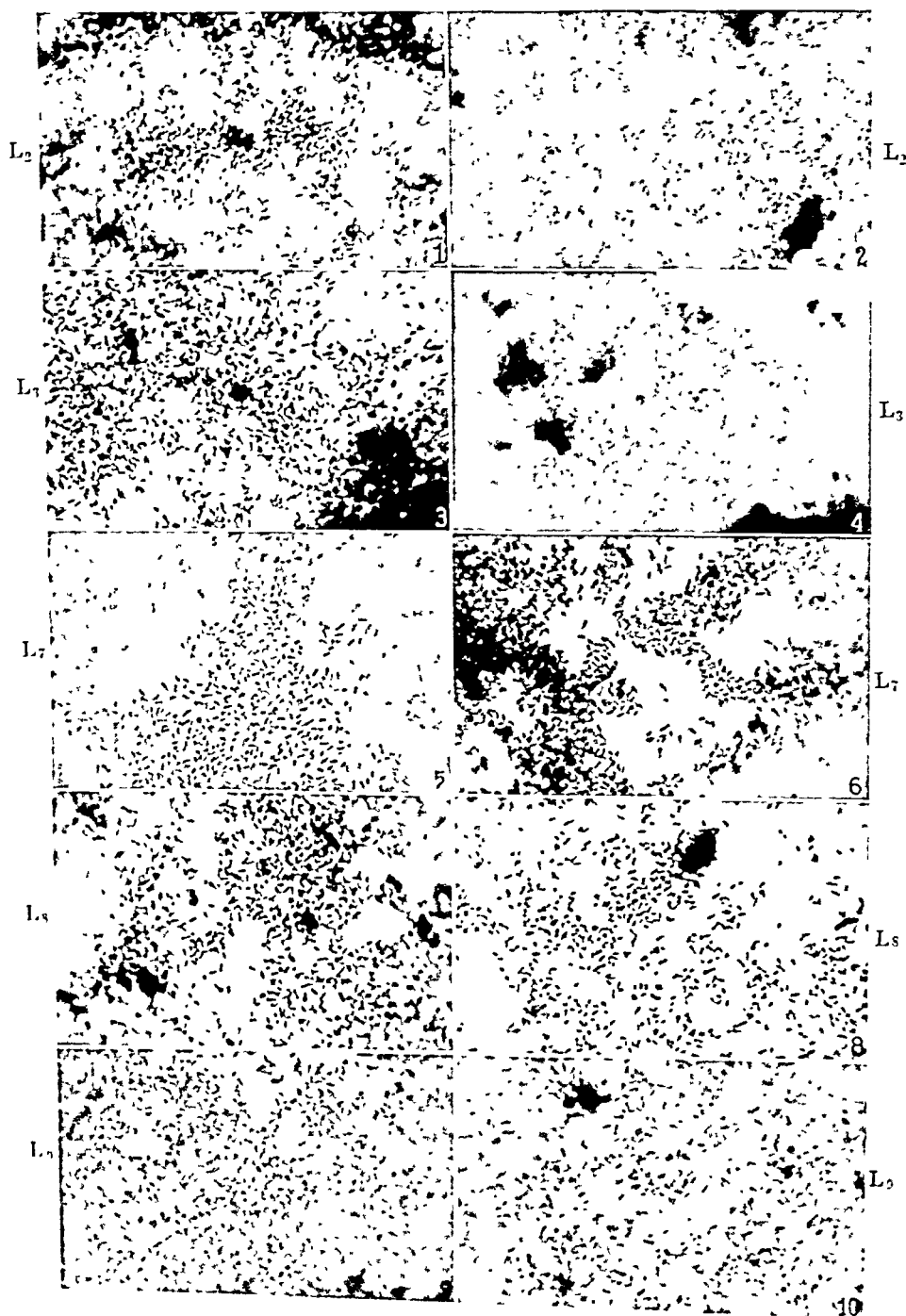
contained live bartonellas at the time they were inoculated, and blood culture revealed the presence of the microorganisms in the blood of monkeys which showed no other signs of infection after inoculation with the human blood. Whether this striking difference is merely a quantitative one or is due to some factor still unknown—such as, for example, a biological phase of the microorganism—has not been determined. The uniformly negative results of transmission experiments with blood by previous investigators is explained by an actual inability of the blood to induce skin lesions and the lack, until now, of a reliable method of detecting *Bartonella bacilliformis* in the monkeys' blood.

The strains isolated showed similar serologic properties, as tested by complement fixation.

Comparison of different strains of *Bartonella bacilliformis*. $\times 1000$.

Giemsa's stain.

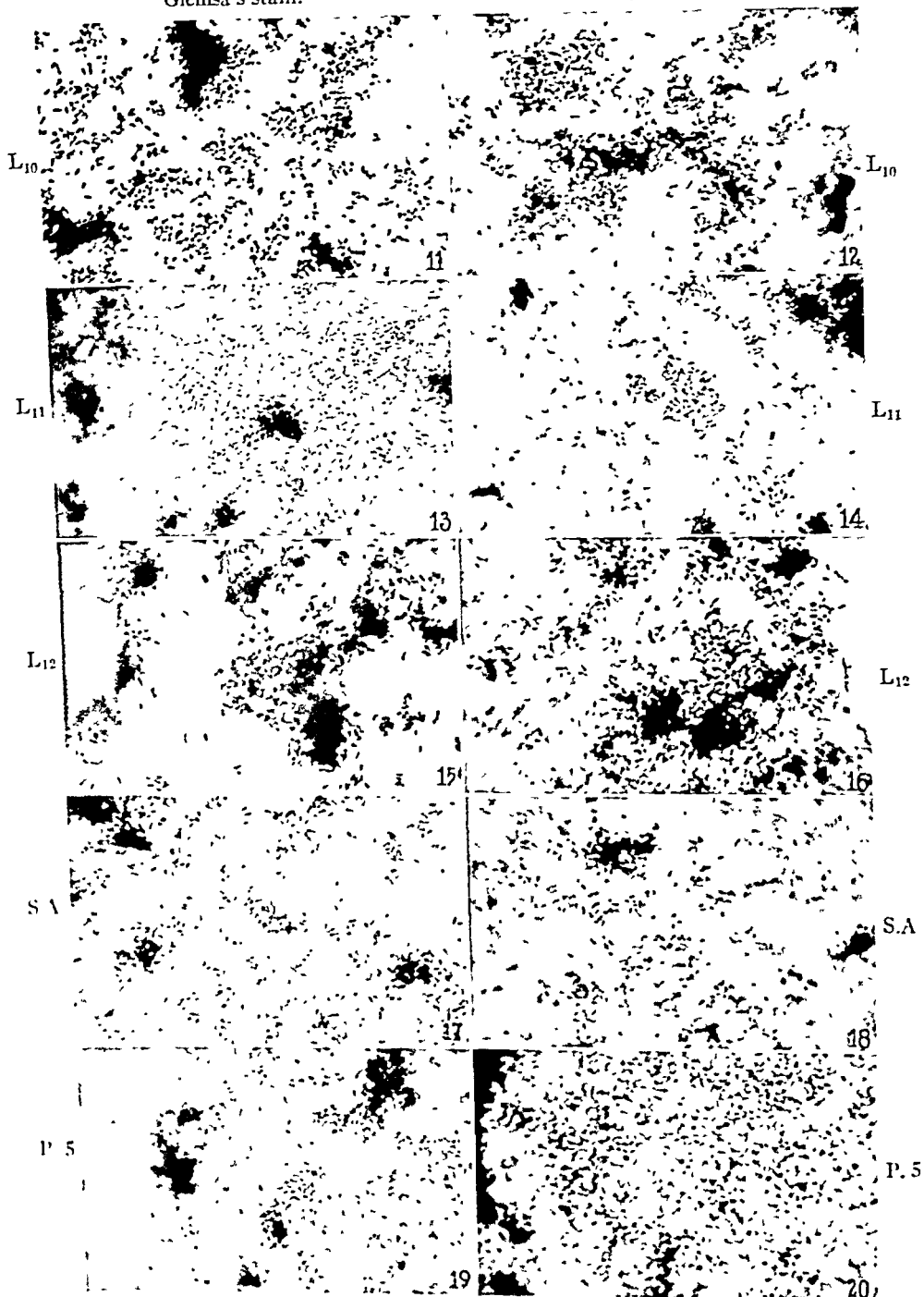
Gram's stain, counterstained.



Comparison of different strains of *Bartonella bacilliformis*. $\times 1000$.

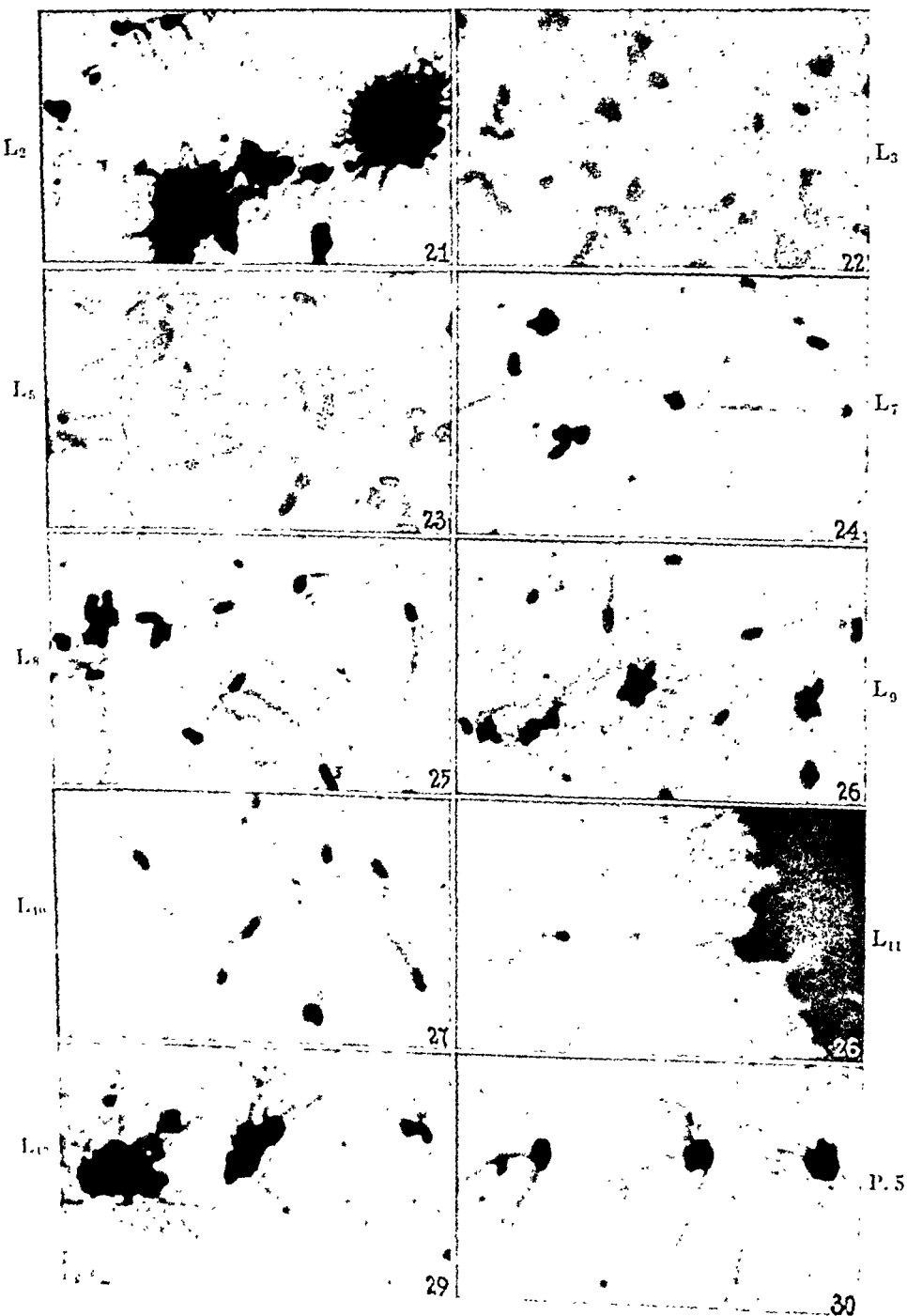
Giemsa's stain.

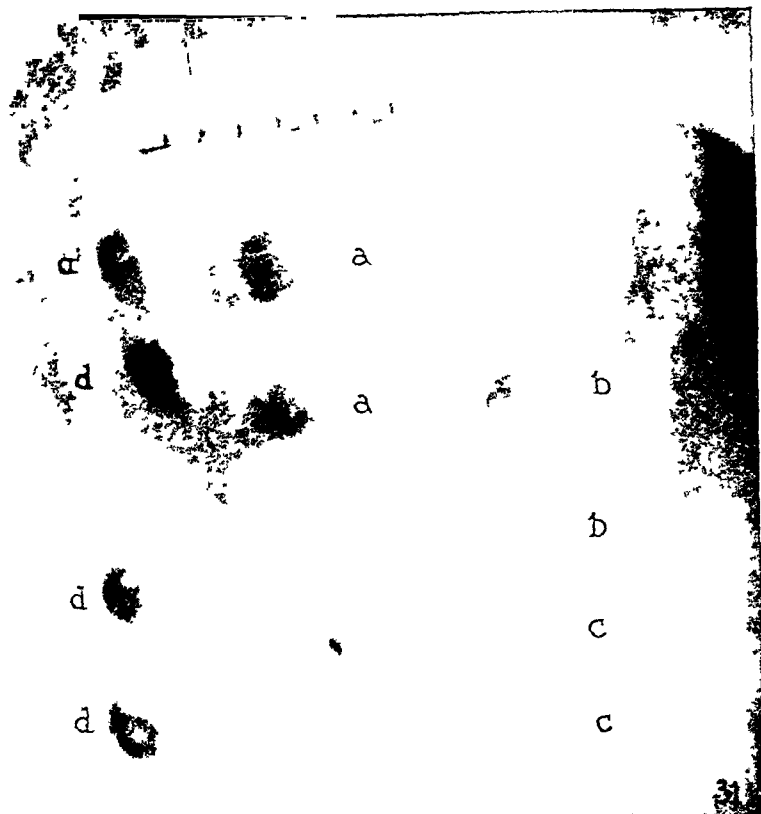
Gram's stain, counterstained.



Comparison of different strains of *Bartonella bacilliformis*. $\times 2000$.

Zettnow-Fontana stain.





Lesions produced by cultures of (a) Strain 2, (b) Strain 3, (c) Strain 5, and (d) Strain P 5. 30 days after inoculation. *M. rhesus* 3A. Natural size.



Abnormal detached flagella. Dark field $\times 1000$

ETIOLOGY OF OROYA FEVER.

XI. COMPARISON OF *BARTONELLA BACILLIFORMIS* AND *BARTONELLA MURIS*. CULTIVATION OF *BACTERIUM MURIUM*, N. SP.

By HIDEYO NOGUCHI, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

PLATES 11 and 12.

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Mayer,¹ in 1921, discovered, in the red blood cells of a rat which was suffering from severe anemia as a result of trypanosomiasis and chemotherapy, peculiar inclusion bodies, resembling *Bartonella bacilliformis* of Oroya fever, which he designated *Bartonella muris*. Subsequently Lauda² reported that splenectomy of white rats brought about a severe anemia which could be transferred to normal rats by inoculating them with the blood or a suspension of liver tissue of the splenectomized animals. The name "infectious anemia of the rat" was introduced to describe the condition. Mayer and his collaborators³ suspected that the anemia resulting from extirpation of the spleen might be due to *Bartonella muris* and succeeded in demonstrating the inclusion bodies in splenectomized rats. Their assumption was that *Bartonella muris* is present in most rats, and that the invasion of the blood is made possible by the removal of a defense organ, the spleen.

Because of the close analogy between *Bartonella muris* and *Bartonella bacilliformis*—their morphological similarity, their localization in the erythrocytes, and their association with a severe form of anemia, a comparative study has been made of these two bartonellas, the specific subjects of inquiry being: (1) the percentage incidence of appearance of *Bartonella muris* in the blood of splenectomized rats and mice, (2) the result of splenectomy in animals other than rats

¹ Mayer, M., *Arch. Schiffs- u. Tropenhyg.*, 1921, xxv, 150.

² Lauda, E., *Arch. ges. Physiol.*, 1925, cclviii, 529.

³ Mayer, M., Borchardt, W., and Kikuth, W., *Klin. W'och.*, 1926, v, 559.

and mice, (3) the transmissibility of *Bartonella muris* to normal rats, (4) the transmissibility of *Bartonella muris* to other animals, especially splenectomized animals, (5) the susceptibility to *Bartonella bacilliformis* of rats and other animals after splenectomy, (6) the comparative morphological features of *Bartonella muris*, *Bartonella bacilliformis*, and *Grahamella talpæ*, the latter an intracorpuseular parasite of moles described by Graham-Smith,⁴ Thomson,⁵ and Brumpt,⁶ and (7) the cultivation of *Bartonella muris*.

Splenectomy was performed on fifty white rats, three wild rats, three Chinese hamsters (*Cricetus griseus*),⁷ and nine white mice. *Bartonella muris* appeared after splenectomy in the blood of all, in some numerous, in others sparsely. Splenectomy always gives rise to an increase in the number of blood platelets, some of which showed very much more distinct chromatin rods and granules than the platelets of normal animals. These loosely packed or partially liberated thrombocyte bodies are sometimes very easily confused with *Bartonella muris*, especially when lying over or under the erythrocytes.

Splenectomy was carried out also in eight *Macacus rhesus*, two Java monkeys, four rabbits, and four guinea pigs. No intracorpuseular elements resembling *Bartonella muris* or *Bartonella bacilliformis* appeared in the blood of any of these animals, nor did the animals show any anemia comparable to that observed in the splenectomized rats or mice.

Transmissibility of Bartonella muris.

Repeated attempts have been made to reproduce the anemic condition and cause the appearance of *Bartonella muris* in the blood of normal rats and mice by injection of blood or suspensions of liver tissue (Lauda's method) from splenectomized animals, intravenously, intraperitoneally, intradermally, or intratesticularly. Such experi-

⁴ Graham-Smith, G. S., *J. Hyg.*, 1905, v, 453.

⁵ Thomson, J. D., *J. Hyg.*, 1906, vi, 574.

⁶ Brumpt, E., *Bull. Soc. Path. Exot.*, 1911, iv, 514.

⁷ I am indebted for these animals (which were obtained primarily for other experiments) to Dr. Carl TenBröeck, who procured them for me in Peking, and to Dr. Amos Wong, who was so kind as to bring them to the United States in his personal care.

ments have always been negative in this respect. Nor has it been possible to transmit the condition to splenectomized monkeys, rabbits, or guinea pigs.

In some instances, however, a quite different condition was induced.

In Rats 54 and 55, inoculated intratesticularly with a suspension of the liver of Rat 40, which had shown a marked blood invasion by *Bartonella muris*, the testicles became acutely inflamed. Rat 54 was castrated on the 6th day of disease. The condition persisted in Rat 55 for 10 days, but by the 14th day the testicle had healed. *Bartonella muris* was not found in the blood of either of these animals. Histological study of the excised testicle of Rat 54 showed acute infiltration of polymorphonuclear leucocytes into the acini, together with edema, congestion, and focal necrosis. Rather coarse bacilli were present in the lesion, some having been taken up by the leucocytes. From the testicular tissue was obtained a pure culture of a microorganism to be described later (Strain 54).

Two normal rats, 56 and 57, inoculated intraperitoneally and intratesticularly with the suspension of testicular tissue of Rat 54, became very ill within 48 hours, and in the blood of Rat 56, taken on the 3rd and 4th days, were found a few intracorpuseular bodies suggestive of *Bartonella muris*. The animal died before the 5th day. Rat 57 showed similar elements in the erythrocytes on the 3rd day but none thereafter. It died of diarrhea in 10 days. There was only slight induration of the testicles in these two animals.

A similar result was obtained in one of three normal rats inoculated with citrated blood of splenectomized Rat 41 (*B. muris* ++). In this case (Rat 66) a bilateral intratesticular inoculation had been made. The inflammation of the testicles reached its maximum on the 5th day and receded during the 3 following days. The same microorganism as had been isolated from Rat 54 was obtained from the aspirated fluid (Strain 66). In the other two animals (Rats 64 and 65), in which the inoculation was unilateral, there was no induration. Examination of blood films of these three animals failed to reveal the presence of *B. muris*. Three other normal animals inoculated intratesticularly and intraperitoneally with a suspension of the liver of Rat 41 showed neither testicular induration nor invasion of the blood by *B. muris*.

There is apparently present in the blood or liver of some splenectomized rats a microorganism capable of setting up acute orchitis in normal rats. The transient appearance of intracorpuseular bodies in the two rats inoculated with the testicular tissue of Rat 54 was far from convincing evidence of transmission of *B. muris*. The failure of most of the attempts to transmit the organism shows at least that regular transmission is not readily accomplished in American rats.

Mayer,⁸ in Hamburg, encountered a similar difficulty. It would, indeed, seem unusual that a microorganism which is unable to invade the blood until the spleen is removed should acquire the power to infect the blood of a normal animal by transfer from the splenectomized one.

It has been found that the intraperitoneal deposition of a small fragment of normal rat or mouse spleen, or the intraperitoneal injection of a saline suspension of the tissue, into splenectomized rats or mice will cause the rapid disappearance of *Bartonella muris* from the blood. Hence *B. muris* is extremely sensitive to splenic substances, even when the spleen itself is not present.

Susceptibility to Bartonella bacilliformis of Splenectomized Animals.

Experiments to be reported elsewhere have shown that the spleen has no important defensive function against the invasion of *Bartonella bacilliformis*. While the blood culture titers of splenectomized monkeys infected with *Bartonella bacilliformis* were usually higher than those of control animals of the same series, yet variations in blood titer are so common in monkeys infected with this microorganism that they cannot be taken as significant in this instance. Rabbits, guinea pigs, and rats were not any less resistant to infection with *Bartonella bacilliformis* after splenectomy than are these animals normally.⁹

Comparative Morphology of B. muris, B. bacilliformis, and Grahamella talpæ in the Blood (Plate 11).

Comparison of blood films stained with Giemsa's solution shows *B. muris* (Fig. 2) to be smaller than *B. bacilliformis* (Fig. 1). There is also less variation in form in *B. muris*, which usually appears as short rods or coccoid forms whose size varies within a very small range. *B. bacilliformis*, on the other hand, though often having an oval or coccoid form similar to that of *B. muris*, frequently occurs in long, slender rods, and Y or V forms are very common (Fig. 1). The long slender forms usually have square ends, as though the or-

⁸ Mayer, M., Borchardt, W., and Kikuth, W., *Arch. Schiffs- u. Tropenhyg.*, 1927, xxxi, 295.

⁹ Noguchi, H., *J. Exp. Med.*, 1927, xliii, 851.

ganism had been cut transversely. Shorter rods may be bent at a sharp angle at one or two places, showing that they are composed of two or three individuals. These branching forms are not found in preparations of *B. muris*, which resembles *Grahamella talpæ* more than it does *B. bacilliformis*, though differing from *Grahamella* in its manner of distribution. I am indebted to Dr. A. C. Coles, of Bournemouth, England, for Giemsa-stained preparations of the blood of two English moles showing *Grahamella* infection (Fig. 3). The grahamellas are often found in such numbers in a single cell as to fill the stroma completely; *B. muris* is usually scattered among many cells, and many individuals are found outside the cells. The preparations show two types of *Grahamella*, one distinctly and uniformly thick, as compared with *B. muris*, and suggesting a bacterium, the other more delicate and showing beaded formations. No branched forms or long threads have been seen in the films of *Grahamella*.

B. muris, *B. bacilliformis*, and *G. talpæ* have similar staining reactions with Giemsa's solution, but basic fuchsin, while giving sharp definition of *B. bacilliformis*, fails to stain *B. muris*.

B. muris of the rat is indistinguishable from the similar elements which appear in the blood of wild rats or Chinese hamsters after splenectomy. The forms found in the corpuscles of splenectomized mice are more granular and show beaded formations.

Cultivation of B. muris.

The etiologic rôle of *B. muris* in the anemia of splenectomized rats is difficult to establish because of the fact that no animal has so far been found which is susceptible to the organism under natural conditions, *i.e.*, when the spleen is present. Once the spleen is removed, *B. muris* appears in the blood, hence splenectomized animals cannot be used for demonstration of the pathogenic effects of a microorganism which has been isolated.

In the course of repeated attempts at cultivation, I have isolated two different organisms, both of which resemble *B. muris* in morphology. The first (Strain 28 A, Figs. 7 and 8) came from the blood of a splenectomized rat and grew on leptospira medium as a grayish layer at the surface. It did not grow on ordinary culture media. It was

subsequently found that this organism had the features of a minute diphtheroid. It had no pathogenicity whatever for normal rats.

From the blood of the same animal (Rat 28) another minute, apparently non-motile, Gram-negative bacterium was isolated on a blood agar plate (Strain 28 B, Figs. 4 to 6). This organism grows on blood agar in very minute colonies but not on leptospira medium or on ordinary culture media. On a blood agar slant it grows more readily, and after 3 or 4 days at 30° or 37°C. the dew-like discrete surface colonies gradually spread to a diameter of 3 mm. or more and, when the surface is densely seeded, coalesce to a shiny, faintly bluish gray, moist layer of growth. The condensation water becomes slightly turbid. This organism is not an acid-producer like the first, but is hemolytic. Morphologically, especially when stained for a short time (20 minutes) with Giemsa's solution it appears very much like *B. muris*. The most interesting feature of the organism is its ability to set up an acute orchitis in normal rats when intratesticularly injected.

The first experiment was made on Rat 42, into which a 24 hour culture grown on blood slants at 30° and 37°C. was injected intraperitoneally and intratesticularly. *B. muris* did not appear in the blood, but the testicle became indurated within 6 days, when the animal was killed for transfer. A suspension of the testicular tissue was used for cultivation experiments and for intraperitoneal and intratesticular inoculation of two normal rats, 52 and 53. A pure culture of the organism was isolated from the testicular tissue. No induration followed the intratesticular injection of the tissue suspension, but in the blood of Rat 52 a few forms resembling *Bartonella muris* were detected 8 days after the inoculation.

The virulence tests of Strain 28 B were repeated on two normal rats, 58 and 59, the cultures being injected intratesticularly only. An acute orchitis developed in both animals, and they died 10 and 9 days after inoculation, respectively. *B. muris* was not found in the blood at any time. From the testicles of Rat 59 pure cultures of the organism were recovered.

Pure cultures of Strain 28 B from Rat 42 were tested on two normal rats, 60 and 61, the injections being made directly into the testicles. Rat 60 reacted more vigorously than Rat 61, the inflammation of the testicle reaching its maximum in 3 days and disappearing in 9 days, while Rat 61 showed only a slight induration on the 3rd day. Bodies resembling *B. muris* were found in very small numbers in the blood of Rat 61, but not in Rat 60. A saline suspension of the testicular tissue of Rat 42, kept at 4°C. for 7 days, was injected into two normal animals, 62 and 63, but in neither was there more than a very slight induration of the testicles. *B. muris* was not found in the blood.

Strain 28 B proved, therefore, to be identical in pathogenicity and cultural properties with the strains isolated from Rats 54 and 66. All three pathogenic strains are evidently the same microorganism. The culture forms are small, measuring 0.4 to 0.8 μ in length and 0.2 to 0.3 μ in width, and appear very much like *Bartonella muris*. The organisms found in the indurated testicular tissues were coarser

TABLE I.
Summary of Experiments with the Organism Cultivated.

Source of culture	Strain No.	Result of inoculation
Blood of splenectomized Rat 28	28 A	Non-pathogenic
Blood of splenectomized Rat 28	28 B	Induced testicular lesion in Rat 42. Culture recovered from testicular tissue Suspension of testicular tissue induced slight induration of testicles in Rats 62 and 63
Testicular tissue of Rat 42, inoculated with culture of Strain 28 B	28 B	No testicular lesions in Rats 52 and 53. Few intracorpuseular elements suggestive of <i>B. muris</i> seen in blood of Rat 52 Induration of testicles in Rats 58, 59, 60, 61. Culture recovered from Rat 59
Testicular tissue of Rat 54, inoculated with suspension of liver tissue of splenectomized Rat 40	54	Slight induration of testicles in Rats 56 and 57. Intracorpuseular elements in small numbers in blood of both
Testicular punctate of Rat 66, inoculated with citrated blood of splenectomized Rat 41	66	Not tested

(1 to 1.5 μ by 0.3 to 0.4 μ), took a deeper stain, and showed very little resemblance to the delicate *B. muris* of the blood of splenectomized rats.

Several normal rats, rabbits, and guinea pigs were injected intratesticularly with pooled cultures of Strains 28 B, 54, and 66 (from Rats 28, 42, 54, 59, and 66). The rats showed the usual marked induration of the testicles, but the other animals reacted only slightly. *B. muris* did not appear in the blood of any of the animals.

Immunization Experiments.

Several normal rats were inoculated intraperitoneally on several occasions with live cultures of Strains 28 B, 65, and 66, and 9 to 10 days after the last injection the animals were splenectomized. Following the splenectomy *B. muris* promptly appeared in the blood of all, that is, previous active immunization with the microorganism in question conferred no protection against the invasion of *B. muris* on subsequent splenectomy.

An immune serum prepared in a rabbit by repeated intravenous injections with live cultures of different strains of the pathogenic microorganism failed to cause the disappearance of *B. muris* from the blood of splenectomized rats or to prevent invasion of the blood by *B. muris* thereafter when given intraperitoneally immediately before splenectomy.

Although neither active nor passive immunity could be induced in rats against *B. muris*, by means of the microorganism under study, this organism is not definitely proven not to be *B. muris* since according to recent observations of Mayer and his coworkers no immunity develops in splenectomized rats as a result of previous infection, the organisms appearing again in the blood of recovered individuals when blood containing them is intracardially injected. In the case of *B. bacilliformis* killed cultures do not constitute a potent vaccine, and an immune serum prepared in rabbits by repeated intravenous injections of live cultures of the organism confers little or no protection against a virulent strain of the parasite, the incidence of takes among vaccinated and control monkeys being practically the same.

The identity of the pathogenic microorganism cultivated from splenectomized rats cannot be established until suitable experimental animals are found. In the meantime I propose for it the provisional name, *Bacterium murium*.

Bacterium murium was not isolated from the blood of 20 normal rats. Its invasion of the blood of splenectomized animals was perhaps due to the removal of the spleen.

SUMMARY.

Bartonella muris appeared in the blood of all white rats, wild rats, Chinese hamsters, and mice, from which the spleen was removed, but

did not appear in that of splenectomized monkeys, rabbits, or guinea pigs.

It has not been possible to transmit *B. muris* to normal rats, monkeys, rabbits, or guinea pigs, by intraperitoneal, intradermal, or intravenous injection of blood containing *B. muris* from splenectomized rats.

In two instances an acute orchitis was induced in normal rats by injection directly into the testicle of blood or saline suspensions of the liver of splenectomized rats. The intracorpuseular elements occasionally found in the blood of some of the animals could not be definitely identified as *B. muris* or as having appeared as a result of the inoculation. The acute orchitis of rats was transferable to normal rats in series.

From the testicular tissue, as well as directly from the blood of a splenectomized rat, there was isolated in pure culture a microorganism which induced in the testicles of normal rats an acute orchitis such as resulted from inoculation of the blood or liver suspensions of splenectomized rats. While a few inclusions were found in the erythrocytes of some of the animals, their number was so small and their occurrence so infrequent that they could not be definitely identified as *B. muris*.

In morphological features the cultural forms of the microorganism isolated resemble *B. muris*. The organism found in the testicular tissues, however, is considerably coarser than *B. muris* and takes a deeper stain.

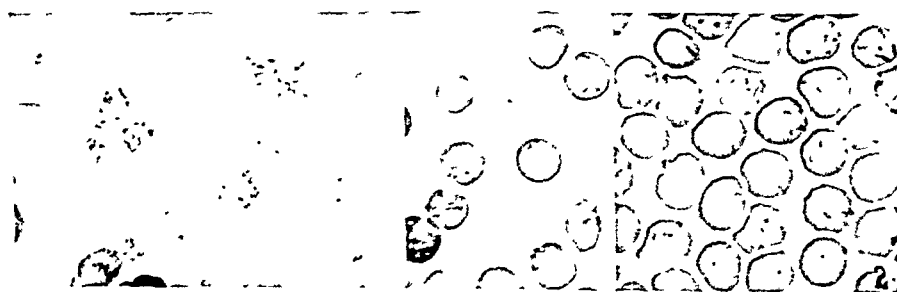
Immunological study failed to settle the question of the relation between *B. muris* and the cultivated organism, which is provisionally called *Bacterium murium*.

Bartonella muris, *Bartonella bacilliformis*, and *Grahamella talpæ* have characteristic individual morphological features.

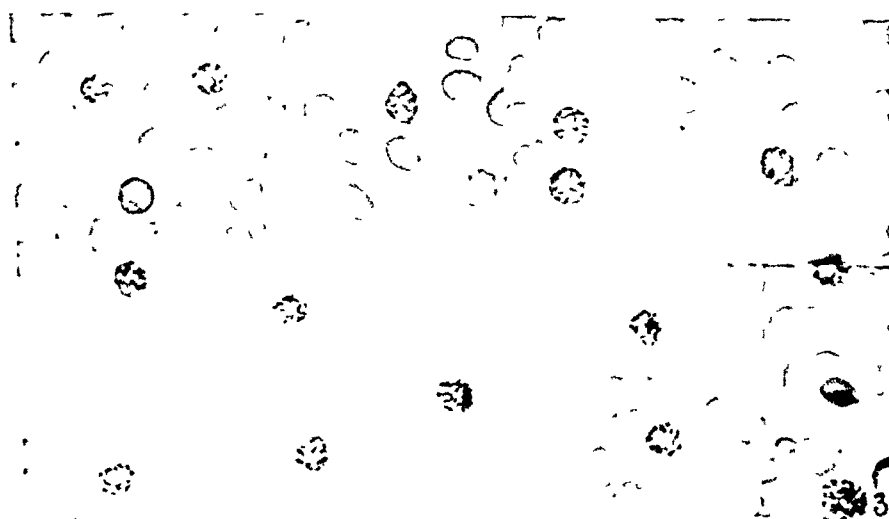
Giemsa's stain. $\times 1000$.



Bartonella bacilliformis in human blood.



Bartonella muris in blood of splenectomized rats.



Leishmania (Leish) in blood of English moles, Coles Strains 1 (above) and 2 (below).



Cultures from blood of splenectomized rats.

4

Strain 28B. Blood agar. 48 hrs. Natural size.



6

Strain 28B. Blood slant. 24 hrs. Dark field $\times 1000$

Strain 28B. Blood slant. 48 hrs. Giemsa's stain. $\times 1000$.



Strain 28A. Dark field. $\times 1000$

Strain 28A. Giemsa's stain. $\times 1000$

THE EFFECT ON SUBSEQUENT AGGLUTINATION OF THE EXPOSURE OF BACTERIA TO HEATED ANTISERUM.

By F. S. JONES, V.M.D.

(From the Department of Animal Pathology of The Rockefeller Institute for Medical Research, Princeton, N. J.)

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Eisenberg and Volk (1) have shown that bacteria treated for intervals of 1 to 2 hours with immune serum heated to 65°C. or 70°C. failed to agglutinate when the heated serum was removed and fresh immune serum added. Ehrlich's conception is that agglutinin is composed of two substances, one that brings about the combination of cell and antibody and another that produces agglutination. His explanation of Eisenberg and Volk's phenomenon assumes that the zymophore substance is changed by heat into agglutinoid which no longer produces clumping. On the other hand, the haptophore portion is unaltered and still combines with the cell to such an extent that further union cannot take place on the addition of unaltered immune serum. This has been accepted as the explanation of the phenomenon.

It seemed possible that Eisenberg and Volk's phenomenon might be explained in other ways. Any serum when heated and added to a bacterial suspension might stick to the surface of the bacterial cell and thus prevent specific union on the subsequent addition of unheated immune serum. Furthermore, it is necessary to show by experiment that the agglutinin or antibody has been completely destroyed when the serum is heated in order to explain the process on the basis of agglutinoid combination. This is especially true in the light of the observations of Beyer and Reagh (2), Orcutt (3), and F. S. Jones (4) that certain types of agglutinin are not destroyed when heated at 70°C. for 20 minutes. A further suggestion is that when too concentrated heated serum is added the reaction may not take place because

of an excess of protein. To answer the questions thus brought up a series of experiments was undertaken.

EXPERIMENTAL.

The agglutinin was prepared by the immunization of rabbits with a motile strain of the hog cholera bacillus and *Bacillus abortus*. The agglutinating serum had been stored in the refrigerator for a month or more. Details of procedure are recorded under the separate experiments.

Experiment 1.—The growth from two 24 hour agar slants of the motile hog cholera bacillus was suspended in 2 cc. NaCl solution. Amounts of 0.3 cc. of this suspension were added to 1.5 cc. of a 1:1 dilution of hog cholera bacillus antiserum which had been heated at 75°C. for 30 minutes; to normal rabbit serum diluted 1:1 and also heated at 75°C.; to normal rabbit serum diluted 1:1; and to 2 cc. of cow serum heated at 65°C. for $\frac{1}{2}$ hour. For control purposes the same amount of culture suspension was added to 1.5 cc. of NaCl solution. All tubes were incubated 1 $\frac{1}{2}$ hours, then 8 cc. of sterile salt solution was added and the tubes centrifuged rapidly. The supernatant liquid was withdrawn and the bacilli resuspended in 10 cc. of salt solution. The suspensions were then tested with hog cholera bacillus serum. Readings were made after 2 hours incubation and refrigeration overnight.

This experiment was repeated with similar results. From the protocol submitted it is evident that exposure to normal serum fails to appreciably affect the agglutinability of the bacilli. However, when the culture was added to the specific serum which had first been heated, the addition of further agglutinin did not materially increase the subsequent agglutination. It is well known from the work of others that 75°C. does not completely destroy the flagellar agglutinin, and as indicated in the control tube, some agglutination had already taken place. During the exposure the contents of each tube were repeatedly agitated by drawing up and rapidly expelling the mixture with capillary pipettes, so that any clumps were readily broken. In addition, after centrifugation the bacilli in the first series were difficult to resuspend and the suspension was relatively unstable since deposition occurred throughout the tubes of the series. It is possible that a little agglutinin still remaining in the serum combines with the bacterial cells, but through mechanical means the clumps may be largely broken so that the bacterial suspension no longer reacts markedly with its antiserum.

It will be noted in the first experiment that heating agglutinin to 75°C. confirmed in a large measure the findings of Eisenberg and Volk. Their experiments were conducted after heating the agglutinin to 65°C. or 70°C. When the lower temperatures were tried with the hog cholera bacillus agglutinin the bacilli were promptly agglutinated, and although the suspensions were agitated vigorously the bacilli settled to the bottom of the tubes after resuspension in salt solution.

It seemed of further interest to observe the effect on subsequent

TABLE I.

The Effect on Subsequent Agglutination of Exposing the Hog Cholera Bacillus to Various Sera.

	Dilutions of unheated hog cholera bacillus immune serum								
	1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:2,560	1:5,120
Bacilli exposed to hog cholera bacillus antiserum diluted 1:1 and heated at 75°C. for $\frac{1}{2}$ hr.	++	++	++	++	++	+	+	+	+
Bacilli exposed to heated rabbit serum	C	C	C	C	C	+++	+	-	-
Bacilli exposed to unheated rabbit serum	C	C	C	C	++	++	-	-	-
Bacilli exposed to heated cow serum	C	C	C	C	C	+++	+	±	-
Bacilli exposed to NaCl solution	C	C	C	C	C	+++	++	+	±

Agglutination has been reported as follows: C, the maximum, with heavy deposit and complete clearing; + + + +, not quite complete; + + +, strong clumping; + +, well defined clumping and a definite deposit in the bottom of the tube; ±, a slight deposit.

agglutination of exposure of the bacilli to immune serum heated at higher temperatures. Experiment 2 covers this phase of the question.

Experiment 2.—The immune and normal rabbit sera were each diluted in 4 parts of salt solution. A portion of each was heated at 75°C. for 20 minutes, another lot was exposed to 80°C. for the same period. To each 2.5 cc. of the heated diluted serum, 0.3 cc. of a heavy suspension of living hog cholera bacilli was added. The same amount of culture was added to NaCl solution for control purposes. All tubes were incubated 1 hour and the contents mixed repeatedly with capillary pipettes. After incubation, 8 cc. of NaCl was added and the mixture centrifuged. The supernatant was then poured off and the bacilli resuspended in 10 cc. of salt

solution. The suspensions were then tested with immune serum. The findings are given in Table II.

It will be noted that the data submitted in the second experiment confirm the first observation. When the bacilli are first submitted to antiserum which has been heated at 75°C. for 30 minutes, they fail to agglutinate to any great extent on the addition of fresh agglutinin. When the immune serum is heated at 80°C. for 30 minutes, the effect is less marked although the agglutinin titer of the unheated serum is appreciably diminished. The effect cannot be ascribed to mechanical

TABLE II.

The Effect of Exposing the Hog Cholera Bacillus to Serum Heated at 75°C. and 80°C.

	Dilutions of hog cholera bacillus immune serum								
	1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:2,560	1:5,120
Bacilli exposed to hog cholera bacillus antiserum diluted 1:4 and heated at 75°C. for $\frac{1}{2}$ hr.	++	+	±	±	±	±	-	-	-
Bacilli exposed to hog cholera bacillus serum diluted 1:4 and heated at 80°C. for $\frac{1}{2}$ hr.	C	C	C	++++	+++	++	-	-	-
Bacilli exposed to normal rabbit serum diluted 1:4 and heated for $\frac{1}{2}$ hr. at 75°C.	C	C	C	C	C	+++++	+	±	-
80°C.	C	C	C	C	C	+++++	+	-	-
Bacilli exposed to NaCl for the same period	C	C	C	C	C	+++++	+	-	-

influences of serum or slowing down of motility, since the organisms exposed to heated rabbit serum or salt solution continued to agglutinate well with the immune serum. Data not given in the table show that immune serum heated at 90°C. for 30 minutes has no appreciable influence on subsequent agglutination.

It seemed of further interest to ascertain to what extent the proteins of the immune and normal rabbit sera actually combined with the bacterial cells. The writer (5) had previously shown that collodion particles and in certain cases bacteria bathed in solutions of various proteins retained sufficient on their surfaces so that they were agglu-

minated on the addition of precipitin specific for the sensitizing protein. With a similar procedure it was hoped that actual combination could be shown.

Experiment 3.—As in the previous experiment, the immune and normal rabbit sera were diluted 1:4. A portion of each was heated at 75°C. and 80°C. for $\frac{1}{2}$ hour. To 3 cc. portions of diluted serum 0.3 cc. of bacterial suspension was added. They were then incubated 1 hour, an excess of salt solution was added, and the tubes centrifuged. The bacilli were then washed twice more in salt solution and finally resuspended in 10 cc. NaCl. The resuspensions were tested with anti-rabbit serum precipitin prepared by injecting a fowl with rabbit serum. The results are given in Table III.

TABLE III.

The Effect of Rabbit Serum Precipitin on Hog Cholera Bacilli First Exposed to Heated Sera.

	Dilutions of rabbit serum precipitin						
	1:50	1:100	1:200	1:500	1:1,000	1:2,000	1:5,000
Culture first treated with antiserum heated at 75°C. for 20 min.	++++	++++	+++	++	+	+	+
Culture first treated with antiserum heated at 80°C. for 20 min.	++++	++	+	±	±	—	—
Culture first treated with normal rabbit serum heated at 75°C. for 20 min.	++	±	—	—	—	—	—
Culture first treated with normal rabbit serum heated at 80°C. for 20 min.	++	±	—	—	—	—	—
Culture carried in NaCl solution	±	—	—	—	—	—	—

From the protocol submitted in Table III, it is evident that when immune serum containing antibody has been heated at 75°C. for 20 minutes, certain of the antibodies are still capable of combining with the bacterial cell and such combination may be detected by precipitin. Thus during union of bacillus and antibody certain of the serum proteins are deposited on the bacteria, and this protein deposit will react with its specific antibody and agglutination result. That there had been considerable deposition of serum protein on the bacilli exposed to the serum heated at 75°C. is clear. It is also true that serum heated at 80°C. is not as efficient in this regard, but nevertheless considerable

must be deposited on the bacterial cell surfaces. The reaction in this instance is well defined, since agglutination occurs when as little as 1/200 cc. of precipitin is added. The control series in which the bacilli were acted upon by normal serum show only slight fixation between bacterium and rabbit serum protein. The bacilli carried in salt solution, but otherwise manipulated in a similar manner, failed to agglutinate when treated with the rabbit serum precipitin.

As a further confirmation of the preceding experiments the following experiment may be briefly cited.

Experiment 4.—When 0.5 cc. of bacillary suspension is added to a suitable amount of complement and 0.5 cc. of antiserum diluted 1:4, and, after suitable incubation, amboceptor and red cells are added, no hemolysis results. The same holds true when the diluted antisera are heated for 20 minutes at 70°C. and 75°C. When the antiserum has been heated to 80°C. there is still considerable diversion of complement (a ++ reaction). When the antiserum is heated to 90°C. even less of the complement is diverted although the hemolysis is not complete.

As the experiment shows, there remains in the serum heated at 70°, 75°, and 80°C. an antibody still capable of combining with its antigen in sufficient quantities to entirely or partially divert complement.

When another organism and its specific serum are substituted for the hog cholera bacillus, essentially the same results are obtained, as will be brought out in Experiment 5.

Experiment 5.—Normal rabbit serum and rabbit serum containing agglutinin for *B. abortus* were diluted in 4 parts of NaCl solution. The diluted serum was distributed in amounts of 3 cc. in a series of tubes, and the tubes were then heated at 70°, 75°, 80°, 85°, and 90°C. for 20 minute intervals. A heavy suspension of *B. abortus* was added to each tube and all were incubated 1½ hours. A tube of bacterial suspension in salt solution was also incubated. The contents of all tubes were agitated at frequent intervals. After incubation, an excess of salt solution was added and the tubes centrifuged. The bacteria were then resuspended in salt solution and tested with unheated specific serum. The results of this treatment on subsequent agglutination are given in Table IV. The controls, where normal rabbit serum was used, failed to show that such treatment influenced subsequent agglutination and are for this reason omitted from the table.

The *Bacillus abortus* serum on the whole behaves when heated much like the other agglutinin. There is however this difference, that the

substance in the abortion bacillus antiserum is more resistant to heat since sufficient combination existed even after heating the antiserum to 80°C. to prevent agglutination on addition of unheated immune serum. Immune sera heated to 85°C. and 90°C. have no inhibiting effect.

That there is an actual fixation between bacterial cell and serum protein can again be shown by noting the effect when rabbit serum precipitin is added to the bacterial suspension which had first been treated with heated agglutinin. This has been brought out in Experiment 6.

TABLE IV.

The Behavior of Bacillus abortus after Treatment with Heated Immune Serum.

		Dilutions of unheated immune rabbit serum							
		1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	Control
Bacilli exposed to diluted serum heated at									
	°C.								
	70	—	—	—	—	—	—	—	—
	75	—	—	—	—	—	—	—	—
	80	—	—	—	—	—	—	—	—
	85	C	C	++++	+++	++	+	+	—
	90	C	C	++++	+++	++	+	+	—
Control—bacilli exposed to NaCl		C	C	C	C	++	+	+	—

Experiment 6.—This experiment is virtually a repetition of Experiment 3 except that *B. abortus* and its agglutinin were used. The procedure was the same. The results are given in Table V.

As in Experiment 3, Table V indicates that antiserum heated at 75°C. actually combines with the bacterial cell in such quantities that agglutination results when a precipitin specific for rabbit serum is added. The blood serum proteins are not dislodged by washing. The combination is less marked when the immune serum is first heated at 80°C., nevertheless the reaction is more intense than that occurring after suspension in immune serum heated at 85°C. and 90°C., or normal rabbit serum heated at the same temperatures.

It was possible by complement fixation tests to further confirm the findings that immune serum heated to 75°C. for 20 minutes completely

deviates the complement in the presence of antigen. That heated to 80°C. markedly deviates the complement, while there is only a slight deviation with serum heated at 85°C., and none with that heated at 90°C.

DISCUSSION.

It is true that bacteria when first treated with immune serum heated at various temperatures may or may not agglutinate on subsequent

TABLE V.

The Effect of Rabbit Serum Precipitin on Bacillus abortus First Treated with Heated Immune Serum.

	Cc. of rabbit serum precipitin					
	1:50	1:100	1:200	1:500	1:1,000	Control
Bacilli treated 1½ hr. with immune serum diluted 1:4 first heated for 20 min. at °C.						
75	+++	++++	++	+	+	-
80	+++	++	-	-	-	-
85	+	+	-	-	-	-
90	+	±	-	-	-	-
Bacilli treated with diluted normal rabbit serum first heated at 75°, 80°, 85°, and 90°C.	+	±	-	-	-	-
Bacilli treated with NaCl solution	-	-	-	-	-	-

exposure to agglutinin. When the hog cholera bacillus antiserum was heated to 75°C. for 20 minutes, the bacilli exposed to its action failed to agglutinate when subsequently treated with unheated immune serum. When the first test serum is heated at 80°C., relatively little inhibiting effect is encountered. The reaction is a specific one, since it is not encountered when normal serum is used in a similar manner. In this regard *Bacillus abortus* acts much like the hog cholera bacillus when similar experiments are performed. However, *Bacillus abortus* serum heated at 80°C. is still capable of preventing further agglutination of the bacilli.

Ehrlich's conception is that two substances produce agglutination,—one is a combining body which is thermostabile, and the other produces clumping and is thermolabile. The latter is converted into agglutinoid

by heat. The combining substance is uninjured by heat and enters into combination with the bacterial cell, thus preventing union on the addition of fresh agglutinin. The evidence that I have submitted while not conclusive is at least suggestive that the reaction may be explained on other grounds.

In the first place, the temperature at which the earlier investigators heated their sera (65°C. and 70°C.) has in my hands always agglutinated the organisms. Furthermore, they worked with undiluted serum and it is possible that agglutination failed to take place because of an excess of colloid, *i.e.* they may have been working in the prozone. When the evidence in regard to the hog cholera bacillus and its agglutinin is analyzed more carefully it is evident that heating at 75°C. for 20 minutes is insufficient to destroy the agglutinin. In fact, the bacilli will agglutinate with such serum provided the incubation is long enough and if interfering mechanical factors are avoided. It has been shown that this is the case by several investigators. The writer (4) brought out the fact that as the temperature increases the agglutinin content declines, at first gradually, until 75°C. is reached. The break is sharp at 80°C., although a little antibody can be detected even after heating to 90°C. There remains, then, in the case of this serum considerable agglutinin after heating the serum to 75°C. It is suggested that this agglutinin combines with the bacterial cell, but as the result of mixing, too short incubation, or for other reasons, the combination is not quantitatively sufficient to produce the phenomenon of agglutination. It may be that agglutinin is so modified during heating at certain temperatures that, although still capable of combining with the organisms, the usual phenomenon of such combination (agglutination) fails to take place. The union is apparently sufficient to prevent further union when fresh immune serum is added. That union actually takes place between the bacterial cell and the serum proteins of certain heated sera is definitely shown by the reaction when a specific precipitin is added, since it is known that agglutination results when bacteria or collodion particles are sensitized to proteins on addition of specific precipitin. As further evidence the behavior of the heated serum in the complement fixation tests is suggestive. In both cases cited the sera heated sufficiently to prevent the secondary agglutination after the addition of fresh serum always deviated the complement. When the sera were heated to higher

temperatures and failed to prevent the secondary agglutination, then relatively little or none of the complement was deviated.

The evidence for *Bacillus abortus* agglutinin is not so clear, although it is known that the agglutinin will resist heating to 70°C. for 20 minutes. Serum heated above this point no longer causes agglutination. Nevertheless when the immune serum is heated to 75°C. and 80°C. it is still capable of preventing a second agglutination. It is clear, however, that actual union of bacterial cell and serum proteins occurs even after heating the immune serum to these temperatures. It is also true that such sera were able to bind complement.

In the light of the experimental evidence the burden of proof that agglutinoid actually exists remains to be established, since the blocking of secondary agglutination can be explained on the basis of residual antibody.

SUMMARY.

It is shown that when dilute rabbit serum rich in agglutinin for the hog cholera bacillus is heated at 75°C. for 20 minutes and the bacilli incubated with the heated serum, agglutination fails to result on the addition of unheated immune serum. When the immune serum is first heated to 80°C., it no longer greatly inhibits secondary agglutination when the organisms are exposed to fresh agglutinin. The abortion bacillus agglutinin acts in a similar manner except that the immune serum must be heated above 80°C. for 20 minutes to prevent the second agglutination. The reactions are specific since control experiments with normal rabbit serum heated at various temperatures failed to influence further agglutination. It has also been shown by precipitation tests that there is definite fixation of serum proteins and bacterial cells with the heated sera which would prevent subsequent agglutination. Furthermore, heated antiserum which would prevent the secondary agglutination still possessed the property of deviating complement in a hemolytic series.

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A COMPARISON OF THE SPIROCHETE OF YELLOW FEVER (LEPTOSPIRA ICTEROIDES NOGUCHI) WITH THE LEPTOSPIRA OF WEIL'S DISEASE.

By ERICH MARTINI, M.D.

(From the Hygienic Laboratory of Samper and Martinez, Bogota, Colombia,
South America.)

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In the year 1923, yellow fever prevailed in Bucaramanga, the capital of the Department of South Santander in Colombia, South America. The diagnosis, which was made at the time by a group of local physicians,¹ was confirmed by a commission of the International Health Board of the Rockefeller Foundation.² This commission conducted its work in our laboratory at Chapinero (Bogota). The confirmation was made serologically by Pfeiffer tests in guinea pigs furnished by us, cultures of *Leptospira icteroides* having been forwarded by Dr. Noguchi. It then became an aim of the laboratory to be prepared to determine a diagnosis of yellow fever by Pfeiffer tests. For the purpose we made an attempt to grow our own cultures of *Leptospira icteroides* from the only guinea pig that survived the Pfeiffer tests of the commission.

The animal (Guinea Pig 1) had been inoculated intraperitoneally by Dr. Pothier on May 16, 1923, with a rich culture of *Leptospira icteroides* (grown on Noguchi medium) together with human serum from a suspected case of yellow fever. On May 24, 1923, 8 days later, heart's blood was taken from the inoculated animal and placed in Ungermann medium, containing rabbit serum inactivated for 30 minutes at a temperature of 58-60°C. and covered with a protective layer of paraffin. On June 9, 1923, the culture showed an abundant growth of lepto-

¹ Drs. Daniel Peralta, Martin Carvajal, Francisco Pradilla Gonzalez, Andres Gomez, Julio Valdivieso, Roberto Serpa, and Louis Ardila Gomez, who in a publication issued in April, 1923, substantiated their findings by clinical histories.

² Drs. J. H. White, Pothier, and Wenceslao Pareja, who were sent in response to a request made by the Government of Colombia, through the Director of Hygiene, Dr. Pablo Garcia Medina.

spira, and we were, therefore, in a position to undertake serological tests for yellow fever.

In our subsequent work we employed the pure or a modified form of the Unger-mann culture medium and adopted the following procedure. During the early months, we made weekly transfers to guinea pigs. Certain animals were inoculated with *Leptospira icteroides* (Noguchi), and others, for purposes of comparison, with *Leptospira icterohæmorrhagiæ* (Weil's disease). Series of cultures were made in both instances, at first with heart's blood, or suspensions of liver and kidney. Later on, cultures were inoculated only with heart's blood. In every case, the pathogenic material was taken on the day of maximal fever and cultured promptly.

With the cultures thus obtained, guinea pigs were again inoculated intraperitoneally, and from the heart's blood of the infected animals, other cultures were made for the inoculation of fresh guinea pigs, and so on. It was observed that the virulence of the spirochetes decreased gradually in the process of passage, so that finally, in August, 1923, after about 3 months of transfer, no animals died of the infection, although large numbers of spirochetes were injected. During the subsequent months, an animal would now and then exhibit a slight degree of the characteristic icterus, but notwithstanding the high fever (up to 41°C.), all survived the infection. Ultimately no jaundice was perceptible, but cultures could still be obtained from the heart's blood taken at the height of the fever. As Lebrede of Havana in his publications reported that he had observed viability up to 7 months of *Leptospira icteroides* in cultures made on the Noguchi medium, we continued to make inoculations of guinea pigs, and subsequent cultures with the heart's blood, at intervals of from 3 to 6 months, and are today—more than 3 years later—still in possession of living cultures of this particular strain.

Leptospira icterohæmorrhagiæ, which by our present methods cannot be distinguished morphologically from *Leptospira icteroides*, was likewise inoculated and cultured. No means of differentiation between the two types of leptospira was apparent at first. But later on, differences in respect to the duration of life of the two types of cultures became evident, and these differences increased as modifications in the culture media were undertaken.

The cultures of *Leptospira icterohæmorrhagiæ* during the early months were grown on Unger-mann medium; later on, we employed for one half of the cultures a medium made of inactivated rabbit serum and physiological salt solution in the proportion of 1: 3; and finally the heart's blood only was used in physiological salt solution, a process which appears to have been first employed by Uhlenhuth and Zuelzer. In a number of instances, cultures were made with rabbit serum which had not been inactivated, but this method was abandoned, since as a rule

the results thus obtained were not as good as when the original Ungermann method was employed.

About 100 cultures of each of the two types—*Leptospira icteroides* and *Leptospira icterohæmorrhagiæ*—were made according to the original Ungermann technique; about an equal number of cultures with inactivated rabbit serum and physiological salt solution, 1:3; and 50 cultures each in physiological salt solution. In a few cases, a 1:3 mixture of uninactivated rabbit serum and physiological salt solution was used. The employment of the last mentioned medium did not give as consistent results as the mixture made with inactivated rabbit serum, although one of these *Leptospira icteroides* cultures proved viable after 2 years and 5 months.

It is noteworthy that when a small quantity of rabbit blood was added to the mixture of rabbit serum and physiological salt solution, the growth of *Leptospira icterohæmorrhagiæ* was abundant, while on the other hand the cultures of *Leptospira icteroides* in this medium did not as a rule show a great increase of growth. But neither of the two types of spirochete was viable for particularly long periods of time when grown in this medium.

Tables I and II show the growth periods of the two types in various media, the longest periods being recorded. The terms *Leptospira icteroides* and *icterohæmorrhagiæ* have been employed because they are current in Central and South America.

The conclusions to be drawn from these tables are:

1. In a medium made of physiological salt solution, in which *Leptospira icteroides* is known to grow less vigorously than *Leptospira icterohæmorrhagiæ*, the longest period of growth for *Leptospira icteroides* was $6\frac{1}{2}$ months, i.e. a period about one-half to two-thirds that of *Leptospira icterohæmorrhagiæ*, which was alive after 11 months.

2. In the Ungermann culture medium, *Leptospira icteroides* lived nearly a year longer (3 years in all) than *Leptospira icterohæmorrhagiæ* (2 years and 1 month).

3. With the use of inactivated rabbit serum and physiological salt solution, 1:3, *Leptospira icteroides* survived six times as long (3 years and $6\frac{2}{3}$ months) as *Leptospira icterohæmorrhagiæ* ($6\frac{2}{3}$ months), although the growth of the latter was abundant.

In judging these cultivation experiments, account must be taken of the fact that a comparison has been made in each case with but a single strain of the two types of leptospira. In order to arrive at

TABLE I.

Leptospira icteroides.

No.	Guinea pig	Dates cultures were made	Medium	Date last examined	No. of surviving cultures	Viability	
						yrs.	mos.
1	2	9/24/24	Isotonic salt solution	11/ 6/24	1		1½
2	3	9/24/24	" " "	11/ 6/24	1		1½
3	4	5/26/24	" " "	9/ 8/24	1		3½
4	5	12/20/24	" " "	5/31/25	1		6½
5	6	5/14/25	Blood; inactivated rabbit serum, isotonic salt solution	3/ 5/26	1		9½
6	7	1/25/24	Inactivated rabbit serum, isotonic salt solution	12/23/24	1		11
7	8	3/12/26	Ungermann	5/27/27	1	1	2
8	2	9/24/24	Inactivated rabbit serum, isotonic salt solution	3/11/26	1	1	6
9	9	9/11/23	Ungermann	5/19/25	1	1	8
10	10	3/10/24	Inactivated rabbit serum, isotonic salt solution	3/ 5/26	1	1	11
11	9	9/11/23	Ungermann	10/ 5/25	2	2	
12	11	12/ 1/23	"	3/ 5/26	1	2	3
13	12	12/22/24	Active rabbit serum, isotonic salt solution	5/27/27	1	2	5
14	13	12/ 1/23	Ungermann	12/10/26	1	3	
15	7	1/25/24	Inactivated rabbit serum, isotonic salt solution	5/27/27	1	3	4
16	14	11/ 5/23	Inactivated rabbit serum, isotonic salt solution	5/27/27	1	3	6½

TABLE II.

Leptospira icterohæmorrhagiae.

No.	Guinea pig	Dates cultures were made	Medium	Date last examined	No. of surviving cultures	Viability	
						yrs.	mos.
1	15	12/20/24	Isotonic salt solution	5/19/25	2		6
2	16	5/26/24	Inactivated rabbit serum, isotonic salt solution	12/17/24	1		6½
3	17	8/22/24	Isotonic salt solution	5/19/25	1		9
4	18	5/26/24	" " "	4/29/25	2		11
5	19	11/30/23	Ungermann	5/19/25	1	1	5½
6	20	11/30/23	"	5/19/25	1	1	5½
7	21	3/10/24	"	3/ 6/26	1	2	
8	22	1/22/24	"	2/26/26	1	2	1

really conclusive results, it will be necessary to make comparative studies of many strains of both types. The above mentioned results, however, may serve to indicate a direction for further investigation, since they suggest a possible means of differentiation of the organisms.

As the course of the infection with *Leptospira icteroides* in the experimental guinea pig has been minutely described by Noguchi, we shall not take it up here. The clinical history and the pathological anatomy of the animals have moreover been extensively dealt with by W. H. Hoffmann of Havana. On the basis of careful experimental work, the last mentioned author maintained for a long time a skeptical attitude regarding the etiological significance of *Leptospira icteroides* in yellow fever, because of the clinical and pathological similarities to *icterohæmorrhagiæ* infections in guinea pigs. Finally, however, even Hoffmann, on the basis of his studies of human yellow fever cases, of the Pfeiffer reaction, and of the specificity of the yellow fever vaccine and serum, appears no longer to doubt that *Leptospira icteroides* of Noguchi is the active, causative agent of yellow fever.

With regard to *Leptospira icteroides* from the guinea pig, the following points which Noguchi has already elucidated appear to be significant:

As a rule, the temperature attains its greatest height (40–41°C. and over) from the 4th to the 7th days after inoculation, while the peak is only occasionally attained on the 3rd day, or on the 8th to 11th days. Sometimes, however, the temperature may go as high as 40°C. on even the 14th day after inoculation. Temperatures up to 41°C. and over, are, however, not necessarily indicative of a severe infection. Guinea Pigs 23, 24, and 11 inoculated with *Leptospira icteroides* recovered completely after very high temperature rises. The first two animals showed the typical icterus. On the other hand, guinea pigs may succumb in a few days with a temperature of less than 40°C., the temperature suddenly falling below normal. In such cases, icterus, typical cutaneous hemorrhages, epistaxis, necrosis of the liver, and lime casts in the kidneys were all noted.

It is desirable to take the material for cultivation—heart's blood, suspension of liver or kidney—at the height of the fever, if possible when the temperature has reached 40° or over, and to place it immediately in the culture medium. According to our experience, this high body temperature, which is most favorable for insuring a vigorous culture growth, continues for only 1 or 2 hours and does not progress after the initial rise has been attained. Particularly is this the case in infections produced with avirulent cultures. Hence it is necessary in such instances to watch the animals closely in order to utilize a favorable opportunity for obtaining blood or other material.

The earliest period at which we were able to obtain positive results from cultures was 5 days after planting. As a rule, however, an abundant growth could not be obtained before about the 10th day. It happened not infrequently that culture media which appeared to be sterile for a number of weeks began to show signs of growth after about a month.

The temperature most favorable to the growth of cultures ranged between 24° and 34°C., but cultures kept at temperatures below 30°C. retained their viability for a longer time, for several weeks at least. If kept between 22° and 24°C., the organisms remain viable for a long period.

CONCLUSIONS.

The most noteworthy point observed in our studies is the extraordinary duration of life and the relatively meagre requirements for sustenance of *Leptospira icteroides*. It is conceivable that under natural conditions opportunities might arise for the prolonged existence of *Leptospira icteroides*, so that possibly after a lapse of years, the disease might reappear without introduction from outside. At any rate, the great viability of *Leptospira icteroides* must be considered in this connection.

A decrease in the virulence of the leptospira does not rule out the possibility of a renewed outburst of yellow fever, since Uhlenhuth and Zuelzer have shown that it is possible to set up Weil's disease in guinea pigs by means of apparently saprophytic spirochetes obtained from drinking water, when the virulence of these organisms has been artificially heightened.

In conclusion the writer takes pleasure in expressing his appreciation for the collaboration in this work of Dr. Bernardo Samper Sordo.

THE RÔLE OF THE RETICULO-ENDOTHELIAL SYSTEM IN IMMUNITY.

V. PHENOMENA OF PASSIVE IMMUNITY IN BLOCKED AND SPLENECTOMIZED MICE.

By C. W. JUNGEBLUT, M.D.*

(From the Department of Bacteriology, College of Physicians and Surgeons,
Columbia University, New York.)

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INTRODUCTION.

The study of the reticulo-endothelial system in its relation to the various phenomena of immunity and anaphylaxis has during the last few years engaged an increasing number of investigators. Several extensive reviews (1-6) on this subject have recently appeared to which the reader is referred for detailed information. In this country the work of Gay and his associates has done much to bring to light the important rôle of the reticulo-endothelial cells in the formation of antibodies, particularly with reference to the mechanism of local immunity. Valuable information on the significance of the reticulo-endothelial system in the various immunity reactions has been derived from an application of the so called blockade method, either alone or together with splenectomy, to the particular problem under consideration. Without discussing the merits of this procedure as a means of eliminating or temporarily invalidating the normal physiological action of the respective cells, let it suffice to state that methods of blockade have been of distinct value in the experience of many workers, although it must be conceded that others have not been able to secure such uniform results.

The problem as to the site of antibody production may be approached (1) by measuring *in vitro* the antibody titer of blocked or splenectomized animals immunized with non-living antigens, and (2) by following the course of various acute and chronic infections in animals so treated. Observations of the latter kind have up to the present been less numerous. Besides the attempts which have been made to break the natural immunity of certain species to given infections and intoxications by endothelial blocking or splenectomy (7), the work of Tsuda (8), Hoen, Tschertkow and Zipp (9), Ledingham (10), Wright (11), Singer and Adler (12), Oerskov (13) and Bass (14) should be mentioned in this

* Fellow in Medicine, National Research Council.

connection. In the case of relapsing fever in mice the apparent lack of antibody production after blockade and splenectomy was recently demonstrated by the writer (15). These findings were fully corroborated by the contemporaneous work of Kritschewsky and Rubinstein (16), and further amplified by the more recent studies of Lisgunova and Butjagina (17). On the other hand, Feldt and Schott (18) and Bruynoghe and Collon (19) did not observe any difference in the course of this infection between blocked or splenectomized and normal mice. The reason for this discrepancy is not altogether clear. That one may not always be justified in generalizing from observations on one type of infection is illustrated by the fact that in some unpublished experiments, the writer noted no material difference in the character or course of syphilitic and tuberculous infection in blocked and splenectomized mice and guinea pigs. This refers to the manifestations and distribution of the clinical lesions in general. Likewise, in rabbits infected with *Trypanosoma equiperdum*, the number of parasites in the blood remained unaltered after the administration of massive intravenous doses of Indian ink. Mention should also be made that the parasites disappeared critically from the circulation on or about the 6th day of the disease—a phenomenon which was described by Mutermilch in 1911 (20)—with the same regularity in blocked animals as they did in controls.

The introduction of blockade methods in the endeavor to analyze phenomena of passive immunity has only recently been made by Neufeld and Meyer (21) and Meyer (22), although the earlier studies of Lippmann (23) had already served to emphasize the significance of the mobile phagocytic cells for the establishment of passive immunity in pneumococcus and streptococcus infection. The first mentioned authors were unable to note any difference between blocked or splenectomized and normal mice with reference to the protective action of antipneumococcus serum against *Pneumococcus* Type I infection in this species. Meyer (22), therefore, concludes that the destruction of the pneumococci by the immune serum is essentially a humoral process, not primarily involving the participation of phagocytosis.

In view of the many facts which suggest the important function of the phagocytic cells, both mobile and sessile, in the elimination of pneumococci from the circulation of the immune animal, it appeared desirable to employ the experimental methods of blockade or splenectomy in a study of the passive immunity against this infection. At the same time it was considered of particular interest to carry on an investigation, based on similar principles, into the nature of passive immunity to tetanus toxin. The disease processes mentioned were chosen on the ground that they represent examples of two types of immunity, that is, antibacterial and antitoxic, which are believed to differ fundamentally in their mechanism.

EXPERIMENTAL WORK.

A. Experiments on the Effect of Blockade and Splenectomy on the Passive Immunity in Pneumococcus Type I Infection.

These experiments were carried out in normal white mice weighing from 18 to 24 gm. The pneumococcus strain was a highly virulent Type I culture (N5); the immune serum, an Antipneumococcus Type I horse serum produced for therapeutic purposes. Both the bacterial strain and the serum were obtained from the Division of Laboratories and Research of the New York State Department of Health, Albany, New York, through the courtesy of Dr. Wadsworth and Miss Kirkbride.

Before studying the effect of blockade and splenectomy on the protective action of antipneumococcus serum against pneumococcus infection in mice, it has seemed advisable, for purposes of control, to obtain data on the influence of the above mentioned experimental procedures on the course of untreated pneumococcus infection in this species. It was found that neither a preceding intravenous injection of India ink (1 cc. of 1:15 dilution) nor the removal of the spleen, nor a combination of both these procedures was capable of altering the course of *Pneumococcus* Type I infection in mice. This statement is made with reference to the size of the minimum fatal dose, the length of time the animals lived and the time of appearance of the pneumococci in the blood stream after intraperitoneal inoculation. Essentially the same conditions prevailed when the intravenous route was chosen as the mode of infection, with the exception, however, that death was very much delayed in both the experimental animals and controls. Doses of 0.000001 cc. and 0.001 cc., if given intravenously, caused death only after 5 and 3 days respectively, while the same doses upon intraperitoneal inoculation killed the animals promptly in from 24 to 48 hours.

The influence of blockade and splenectomy on the action of Antipneumococcus Type I serum in *Pneumococcus* Type I infected mice was studied next. The experiments were carried out by injecting intraperitoneally mixtures of a constant amount of serum (0.1 cc.) and varying doses of an 18 hour culture (0.1, 0.2, 0.3 cc.), in a uniform volume of 0.5 cc. each, into three groups of mice. The first group represented normal controls, the second had received 1 to 2 hours before an intravenous injection of India ink (1 cc. of 1:15 dilution) while the last group had been splenectomized 2 days previously. For each mixture, duplicate animals were run, and the final results of each test were based on an arbitrary 4 day observation. Virulence controls (0.000001 cc. and 0.00000001 cc.) accompanied each test. Instead of recording each individual experiment, the results of five tests, carried out on separate dates, are chartered together. Although there are disadvantages to this method of evaluating the results, it is possible to obtain average figures without permitting the irregularities in virulence and the variability in individual susceptibility to obscure the fundamental results in the experiment.

As may be seen from Table I, the protective action of Antipneumococcus Type I serum against the homologous infection was definitely lower in mice injected with India ink as compared with the normal controls. While 0.1 cc. of serum protected fully 100 per cent of normal mice against 0.1 cc. of culture, only 40 per cent of the blocked mice lived on the 4th day after the test. On the other hand, when splenectomy preceded the test shortly before, there seemed to be no

TABLE I.

*Action of Antipneumococcus Type I Serum against Pneumococcus Type I Infection in Normal, Blocked and Splenectomized Mice.
(Combined Results of Five Tests.)*

No. of animals	Preparation	Mixtures (injected intraperitoneally)		Result		Protection
		Serum	Culture	Survived	Died	
		cc.	cc.			per cent
10	Normal	0.1	0.1	10	0	100
"	Blocked	"	"	4	6	40
"	Splenectomized	"	"	9	1	90
"	Normal	"	0.2	6	4	60
"	Blocked	"	"	3	7	30
"	Splenectomized	"	"	5	5	50
"	Normal	"	0.3	4	6	40
"	Blocked	"	"	1	9	10
"	Splenectomized	"	"	4	6	40
5	Normal	—	0.000001	0	5	0
"	"	—	0.00000001	"	"	"

material diminution in the degree of passive immunity. Similar conditions were found to prevail in the case of other quantitative combinations of serum and culture. In a few instances, the serum was given prophylactically at various intervals (24, 48 and 72 hours) before the infection. Although the results available are too few upon which to base definite conclusions, they indicate that the protective action of a prophylactic dose of serum against subsequent infection was diminished still more in blocked animals.

B. Experiments on the Effect of Blockade and Splenectomy on the Passive Immunity to Tetanus Toxin.

These experiments were carried out in normal white mice, weighing from 18 to 24 gm. The tetanus toxin was a recently prepared broth toxin which at the time of its preparation had a minimum fatal dose for guinea pigs of 0.0001 cc. The antitoxin was a refined and concentrated pseudoglobulin prepared from antitoxic horse plasma, and contained 800 units per cc. Both the toxin and antitoxin were obtained from Dr. Wadsworth and Miss Kirkbride of the New York State Department of Health, to whom my best thanks are due.

In preliminary experiments, the potency of this toxin for mice was titrated in normal animals and in mice which had (1) received one intravenous blocking injection of India ink 1 to 2 hours before the test; (2) which had been splenectomized 2 days previously; (3) which had been subjected to splenectomy and blockade. There was no difference in the lethal action of the toxin for normal and experimental animals, the minimum fatal dose being with each group 0.001 cc. after subcutaneous injection, and 0.0005 cc. after intravenous injection.

The influence of blockade and splenectomy on the neutralization of tetanus toxin by specific antitoxin and on the protection afforded by a preceding dose of antitoxin against subsequent intoxication was studied. The first part of these experiments was carried out by injecting subcutaneously, as well as intravenously, mixtures of a constant amount of toxin (two hundred minimum fatal doses = 0.2 cc.) and varying amounts of antitoxin (0.002, 0.001, 0.0005, 0.00025 cc.) into normal and experimental mice. The mixtures contained the toxin and antitoxin in a uniform volume of 0.5 cc. each and had been held 1 hour at room temperature before the injection was made. Duplicate animals were run for each toxin-antitoxin mixture; the results of two tests are recorded together in Table II.

Table II shows clearly that the various toxin-antitoxin mixtures were toxic or non-toxic for experimental animals to the same extent as they were for the normal controls, the minimum dose of antitoxin which neutralized exactly two hundred minimum fatal doses in this case being in either group 0.001 cc. These results then demonstrate that, after union between toxin and antitoxin has taken place *in vitro*, the reaction on the part of the animal represents merely a registration of the toxicity of a given mixture and the physiological effect is in direct proportion to the amount of free toxin present. This process, in accordance with the previous findings, does not seem to involve primarily the reticulo-endothelial cells in its accomplishment.

Further experiments were planned to provide for a union of toxin

and antitoxin *in vivo*, allowing sufficient time for a general distribution of the antitoxin in the organism. Experimental animals, blocked by an intravenous injection of India ink as described before, together with

TABLE II.

Titration of Tetanus Toxin and Antitoxin in Normal, Blocked and Splenectomized Mice.
(Combined Results of Two Tests.)

No. of animals*	Preparation	Mixtures (injected either subcutaneously or intravenously)		Results
		Toxin	Antitoxin	
4	Normal	cc. 0.2	cc. 0.002	All four alive 4th day
"	Blocked	"	"	" " " " "
"	Splenectomized	"	"	" " " " "
"	Blocked and splenectomized	"	"	" " " " "
"	Normal	"	0.001	" " " " "
"	Blocked	"	"	" " " " "
"	Splenectomized	"	"	" " " " "
"	Blocked and splenectomized	"	"	" " " " "
"	Normal	"	0.0005	2 dead 2nd, 2 dead 3rd day
"	Blocked	"	"	" " " " "
"	Splenectomized	"	"	3 " " 1 " " "
"	Blocked and splenectomized	"	"	2 " " 2 " " "
"	Normal	"	0.00025	All four dead 2nd day
"	Blocked	"	"	" " " " "
"	Splenectomized	"	"	" " " " "
"	Blocked and splenectomized	"	"	" " " " "

* In each group of four animals, two received the respective toxin-antitoxin mixture subcutaneously, two intravenously.

an equal number of normal controls were given a constant amount of antitoxin (0.01 cc.) intraperitoneally, and after varying intervals, *i.e.*, 24, 48 and 72 hours, received by the same route graded doses of toxin (0.2, 0.1, 0.05, 0.01 cc.) in order to determine the degree of passive

immunity. The results, which are shown in Table III, demonstrate a marked difference between the experimental animals and controls.

TABLE III.

Protective Effect of a Preceding Dose of Tetanus Antitoxin against Subsequent Intoxication in Normal and Blocked Mice.

No. of animals	Preparation	Antitoxin injected intra-peritoneally	Toxin injected intra-peritoneally	Interval*	Result	
					Survived	Died
1	Normal	0.01	0.2	24	1	0
"	"	"	0.1		"	"
"	"	"	0.05		"	"
"	"	"	0.01		"	"
"	Blocked	"	0.2	24	0	1
"	"	"	0.1		"	"
"	"	"	0.05		1	0
"	"	"	0.01		"	"
"	Normal	"	0.2	48	1	0
"	"	"	0.1		"	"
"	"	"	0.05		"	"
"	"	"	0.01		"	"
"	Blocked	"	0.2	48	0	1
"	"	"	0.1		"	"
"	"	"	0.05		1	0
"	"	"	0.01		"	"
"	Normal	"	0.2	72	1	0
"	"	"	0.1		"	"
"	"	"	0.05		"	"
"	"	"	0.01		"	"
"	Blocked	"	0.2	72	0	1
"	"	"	0.1		"	"
"	"	"	0.05		"	"
"	"	"	0.01		"	"

* This interval represents the elapsed time between the injection of the toxin and the antitoxin.

While, under the conditions of the test, a prophylactic dose of 0.01 cc. of antitoxin was capable of protecting normal mice against 0.2 cc. of

toxin for fully 72 hours, the blocked mice after an interval of 24 and 48 hours were protected only against 0.05 cc. of toxin and after a period of 72 hours no protection at all was noted against even 0.01 cc., the smallest dose of toxin employed.

DISCUSSION.

In discussing the observations made in this study, the fundamental difference between the reaction of the antibacterial serum, on the one hand, and the reaction of the antitoxin, on the other, with their specific antigens, becomes at once apparent. Both, pneumococcus infection and tetanus intoxication in mice, represent examples in which agents of maximum virulence or toxicity are introduced into a host of maximum susceptibility. Interference with the cellular defensive mechanism of the host, such as is brought about by blockade of the reticulo-endothelial system or splenectomy, under such conditions, is not apt to further decrease this minimum of resistance. In accordance with this consideration, it was found that both disease processes were not altered appreciably in their course by the above mentioned experimental procedures. The results obtained from this part of the work at the same time contradict an objection, which has been raised by some authors against the value of blockade as a means for the selective elimination of the antibody-producing reticulo-endothelial cells. It has been claimed that the injection of foreign colloidal material is a procedure which in a general, non-elective fashion lowers the vitality of the animal and consequently reduces its capacity to respond to an antigenic stimulus. The present experiments show that in processes, the acute course of which does not allow for antibody production, neither blockade nor splenectomy affect the general vitality of the experimental animal, as is the case where other experimental methods are used, such as injection of certain chemical poisons, exposure to actinic rays, removal of the glands of internal secretion, starvation, diet deficiencies, etc.

The action of antibacterial serums in general on their respective infections has long been recognized to involve the active participation of the passively immunized individual in the final destruction of the respective microorganisms, quite apart from any possible direct interaction between antibodies and antigen. Such immune reactions differ

from the toxin-antitoxin neutralization not only in the fact that no deleterious reaction *in vitro* is demonstrable without the participation of living tissue cells or of ferments, but also by their failure to follow the laws of multiple proportions. The mere fact that the protection afforded by an antibacterial serum against the corresponding infection is definitely limited, the protective action of increasing amounts of serum not rising proportionately against increasing doses of culture, suggests the existence in the animal of a third factor, which determines to a large extent the outcome of the test. Furthermore, the importance of such individual disposition is reflected by the apparent irregularities in the results of protection tests with streptococcus and pneumococcus sera, while the respective infections alone are marked by their uniform and regular course. In the case of Pneumococcus Type I infection, it has long been realized that the protective effect of antipneumococcus serum against this infection is not due to any bactericidal action of the serum, but must be linked closely with active participation of the phagocytic cells of the treated animal (bacteriotropins). It is, therefore, to be expected that any interference with the physiological integrity of the reticulo-endothelial system must of necessity lower the protective action of the immune serum. The results obtained in this study, which clearly demonstrate a lowered protective effect of Antipneumococcus Type I serum against Pneumococcus Type I infection in blocked mice, in the writer's opinion, may be conveniently interpreted in conformity with the outlined theoretical considerations. In this connection it is of interest to draw briefly attention to the work of Kritschewsky and Meersohn (24), Kolpikow (25) and Feldt and Schott (18), as well as to our own observations (15), which have shown that the chemotherapeutic effect of salvarsan on relapsing fever and trypanosome infection in mice likewise depends on the presence of an intact reticulo-endothelial system. The writer has suggested that the marked discrepancy in the case of salvarsan between the lack of action *in vitro* and the high parasitocidal power of the drug in the infected animal on the corresponding organisms may possibly be explained by a hitherto unknown function of the reticulo-endothelial cells, which may account for the transformation of the inactive preparation into the active derivative. Earlier observations of Rosenthal and Spitzer (26) on the relation of

the trypanocidal action of human serum to the reticulo-endothelial system would seem to strengthen such an hypothesis.

Contrasting the mechanism of passive immunity, induced by the so called antibacterial sera, with an analysis of the toxin-antitoxin reaction, it would appear that the latter is essentially a humoral process, which does not depend upon an active participation of the host. The animal, under such conditions, simply serves as a passive biological index of the amount of free toxin present, no implication of any phagocytic cells being primarily involved in this reaction. In harmony with the above presumption, it was found, when we inject *in vitro* prepared mixtures of tetanus toxin and antitoxin into blocked mice, that the ensuing reaction was in each instance directly comparable and alike with the corresponding effect in normal animals. Essentially different results, however, were obtained, when the antitoxin was given prophylactically and the degree of passive immunity tested against subsequent intoxication. Under such conditions, it was observed that the passive immunity conferred after an interval of 24 and 48, hours respectively, was much lower in blocked mice than in normal controls, a still further reduction becoming manifest after a 72 hour period. In interpreting this observation it is not clear whether the union of toxin and antitoxin *in vivo* depends upon a preceding fixation or attachment of the antitoxin to the reticulo-endothelial or other tissue cells (*cf.* Levaditi and Muter-milch (27), Hahn and von Skramlik (28)) which would be influenced unfavourably by previous blockade, or whether the lower protection is to be attributed to a more rapid excretion in the blocked animals of the antitoxin, which in this case was intimately bound to the foreign protein (horse serum pseudoglobulin). The observations of Saxl and Donath (29) on the slower elimination from the blood stream of foreign substances in splenectomized or blocked animals, and those of Collon (30), which suggest a prolonged presence of foreign protein in the circulation of rabbits after blockade with trypan blue, may be taken to indicate an inhibiting or retarding influence of blockade on the resorption of the antitoxin by the tissue cells. In the case of passive immunity to diphtheria, Kassowitz (31) and Freud (32) have shown, in addition, that a high concentration of antitoxin in the circulation may under certain conditions be associated with a low content of the

antibody in the tissues, in which case the individual would not be protected. The fact, however, that with the increase of the time interval protection was further diminished in the blocked animals in the writer's experiments, would render an explanation based solely upon an altered distribution of the antitoxin between the tissues and the circulation somewhat questionable. Further insight into these phenomena will not be gained until more is known of the elimination of heterologous antigens and homologous antibodies from the circulation and of the mechanism underlying their final excretion from the body, particularly with reference to changes in permeability of the cell membrane following the injection of blocking substances.

SUMMARY.

1. Blockade of the reticulo-endothelial system by means of one intravenous injection of India ink as well as splenectomy did not alter the course of either *Pneumococcus* Type I infection or tetanus intoxication in mice.

2. The protective action of Antipneumococcus Type I serum against the corresponding infection, as determined by the injection of *in vitro* prepared mixtures of serum and culture, was definitely lower in mice which had received one blocking injection of India ink shortly before the test.

3. Titration of tetanus toxin and antitoxin in blocked and splenectomized mice gave results identical with those obtained in normal mice, if *in vitro* prepared and incubated toxin-antitoxin mixtures were injected. The degree of protection, however, conferred by a preceding dose of antitoxin against subsequent intoxication, was markedly lower in blocked mice than in normal control animals, this difference becoming more pronounced with the increase of the time interval.

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THE EXTENT OF THE CAPILLARY BED OF THE HEART.

BY JOSEPH T. WEARN, M.D.

WITH THE TECHNICAL ASSISTANCE OF LOUISE J. ZSCHIESCHE.

(From the Thorndike Memorial Laboratory of the Boston City Hospital, and the Department of Medicine, Harvard Medical School, Boston.)

PLATES 13 TO 16.

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This investigation was begun with the object of determining the relative number of capillaries in normal human hearts as contrasted with the number in abnormal hearts from patients who had shown clinical signs of cardiac failure. In order to approach the problem it was first necessary to establish the size of the capillary bed and the distribution of the capillaries in normal hearts. The present paper, therefore, deals with the capillaries of the normal myocardium as determined by injections through the coronary arteries in man, cats and rabbits, and with the methods used in making these injections.

The coronary vessels have held the attention of numerous investigators for the past 3 centuries. Earlier workers studied their distribution by careful tedious dissections; and during the last few decades the vessels have been injected and outlined with various dyes and masses which have made possible a detailed study of the finer branches. Unfortunately, however, the injection masses used in most instances have failed to penetrate the capillaries and, as a result, the existing knowledge of this important group of vessels is very meagre. The same could be said, moreover, of the capillaries elsewhere in the body until the recent studies of Dale and Laidlaw (1) and Dale and Richards (2) on the effect of histamine upon the circulation, demonstrated the enormous capacity of the capillary bed. Krogh (3) subsequently directed attention to the number and behavior of capillaries in skeletal muscle, and, by his brilliant investigations, opened a new pathway to this extensive but obscure branch of the vascular tree. In 1919, Richards (4, 5) made direct observations upon the kidney of a living frog and was able to study the behavior of the capillaries in that organ.

The heart does not permit of direct observation of the circulation in its walls, although Drury and Smith (6) have observed the blood flow in the branch of the coronary artery which supplies the first part of the pulmonary artery in the

turtle. Attempts to inject the capillaries of the heart have met with indifferent and not altogether uniform results. No effort has been made to determine the relative number of capillaries in the heart and very little study has been directed to their relationship to the muscle fibres, their anastomoses with one another or to their connections with the Thebesian circulation. One worker only, A. V. Meigs (7), injected two hearts with Berlin blue and his study led him to the conclusions that the capillaries in the heart were much larger than those elsewhere in the body and were unique in that they actually pierced and entered the cardiac muscle fibres. The sections of heart muscle reproduced in his paper, however, show an incomplete injection of the capillaries, and his evidence is not wholly convincing. Many other workers have failed to fill the capillaries and Nussbaum (8) was led to say that complete injection of the capillaries of the heart was impossible.

Methods.

Several methods for injecting the capillaries have been employed in the course of the present work, some of which have been successful in a few instances only, while one method in particular has been satisfactory in that it has given a uniformly and constantly complete injection of the capillary bed. Both the successful and unsuccessful methods will be described, the former in some detail, inasmuch as many of the experiments which were regarded as unsuccessful and resulted in incomplete injections of capillaries later proved to be of considerable value in a study of the Thebesian vessels.

In selecting materials for the injection it was necessary to choose substances that were soluble or, if in suspension, with particles of such diameter that they would enter the capillaries without difficulty. The other requirements for the injection material were that it remain in the capillaries, show no extravasation and withstand the process of fixation, embedding and staining. Berlin blue and India ink¹ have been employed frequently for injecting blood vessels and in this investigation they proved to be the most satisfactory of the various substances in suspension. Solutions of diazine green and trypan blue were used in some experiments with success, and many other dyes were tried but with disappointing results.

The first observations were made in hearts removed from cats after death and the method of making the injections was that which

¹ Either Weber's or Higgins' India ink was used.

had been commonly employed by most investigators in studying the coronary circulation.

The hearts were kept in the ice box for 48 hours post mortem in order to allow rigor to pass off. In preparation for injection they were perfused with 0.85 per cent salt solution until the perfusate came through practically clear and the heart appeared to be free from blood. This perfusion was carried out at various pressures ranging from 40 to 250 mm. Hg and at a temperature of 45°C. In some of the experiments the temperature was kept constant at 37°C. After washing out the blood with the perfusing fluid, the injection was made at the same temperature and pressure. A 2 per cent suspension of Berlin blue or India ink diluted with an equal part of distilled water was found to be the most satisfactory injection material. Both the saline solution and the injection suspensions were filtered before using in all experiments to be described hereafter. It was soon found, however, that this method, even when most carefully controlled, failed to inject all the capillaries. When the method was applied to human hearts, it proved even less efficient, and in many instances very few of the capillaries were filled. This failure was due in part to the escape of the dye through the Thebesian vessels.²

The same procedure was carried out on hearts obtained immediately after death with results slightly more encouraging but far from satisfactory.

In some of these experiments potassium sulfocyanate (3 per cent) was perfused through the coronary arteries to prevent constriction of the vessels, but this addition contributed little or nothing to the success of the method. The results were not dependable, for in some hearts scarcely a dozen capillaries per low power field were injected, while in others there were areas where many of the capillaries were filled.

After many other methods had been tried, partial success was obtained by omitting the washing out of the vessels, and injecting the dye without this preparation. Kerr and Mettier (9) found this procedure satisfactory in their study of the vascularity of the heart valves. The results obtained with this method, though better than the first, were not successful in that all the capillaries of the myocardium were not filled. A possible explanation of the variation in the results of this method was furnished in a series of experiments dealing with the Thebesian vessels. In brief, it was found that India ink, injected into the coronary arteries, escaped for the most part (60 to 90 per cent) through the Thebesian vessels and the capillaries remained unfilled. If, on the other hand, there was scant flow from the Thebesian veins and most of the ink escaped through the coronary sinus

² The part played by the Thebesian vessels is included in another paper in this issue.

and veins, the capillaries were apt to be completely or almost completely filled. This method, therefore, in some instances, gave beautiful injections, but it was not dependable and for this reason it was not adopted as the method of choice (see Figs. 1 and 2).

Attention was next directed to living hearts, and the first series of experiments was performed upon cats and rabbits anesthetized with sodium barbital. They were carried out in the following manner.

After complete anesthesia had been obtained, a tracheotomy was done and artificial respiration instituted. The lower part of the sternum was removed with enough of the ribs to expose the entire pericardial sac. Careful hemostasis was practiced and the structures handled as little as possible. Finally, after opening the pericardium in such manner as to bring the left auricle and ventricle into view, the dye was injected through a needle into the left auricular appendage, at a pressure very slightly in excess of that within the auricle. India ink or a dye was injected until the heart showed complete injection grossly, and this after some practise was determined by the appearance of the heart. If ink were used, for instance, the heart became uniformly black only when the capillaries were filled. At this point of maximum filling of the vessels some substance, such as formalin or alcohol, was injected through the same needle in an effort to stop the heart.

The following protocol, which represents the only successful experiment by this procedure, is illustrative.

Experiment Cat 1.—Weight of cat 3 kilos. May 14, 1924. Anesthesia was induced by means of sodium barbital (0.6 gm. per kilo intraperitoneally). Blood pressure was then registered by means of a cannula in the carotid artery. The chest was opened under artificial respiration and the pericardium incised in such a manner that the left auricle and ventricle came fully into view. India ink diluted with an equal part of distilled water and at a temperature of 37°C. was then injected directly into the left ventricle. As the injection began the aorta was clamped at the level of the right carotid artery. The pressure used in injecting the ink was barely sufficient to cause the ink to enter the ventricle against ventricular pressure. When the heart began to turn black, showing a capillary injection, 5 mg. of histamine was injected with the ink. The total amount of ink used was 20 cc. and the injection was made at such a rate that the blood pressure never rose above the original level.

At a certain time during the injection the heart turned very black and at this point a large clamp was placed on the heart in the auriculoventricular groove. The vessels at the base were also clamped, the heart excised and placed in 10 per cent formalin with the clamps in place. This heart ceased to beat almost immediately after the clamp was placed in the auriculoventricular groove. The injection grossly appeared to be perfect.

The experiment reported, however, together with another similar one, Fig. 3, furnishes the only two completely injected hearts obtained thus far in intact animals by injections made directly into the heart with Berlin blue and India ink. The obvious reason why these were successful is that the heart was stopped at the time of the maximum injection of the capillaries. In every other experiment attempts to bring about instantaneous stoppage were unsuccessful³ and as a result the heart, by continuing to beat, "milked out" the dye from its capillaries. This phenomenon held true when the injected material was a suspension such as India ink or Berlin blue, for in many instances small particles of dye or ink adhered to the capillary walls and outlined them very faintly.

The method which finally gave the most satisfactory and almost uniformly successful injections was found to be the perfusion of the isolated beating heart. The method in brief was carried out in the following manner.

Rabbits or cats were killed instantly by a blow on the head. The heart was excised and a cannula tied into the aorta at a sufficient distance from the aortic valves to prevent blocking of the openings of the coronary arteries. The perfusion was immediately started at a pressure of 45 cm. of water, the temperature being kept at 37°C. The perfusate used was oxygenated Locke-Rosenheim solution (10). The chemicals used in the solution were the purest to be obtained upon the market. The water used was distilled from sodium hydrate, the entire distilling apparatus being made of Pyrex glass. All cannulae and apparatus employed in the experiment were thoroughly cleaned and finally rinsed with the glass-distilled water before using.

With some practise a heart can be excised and the perfusion started before the heart ceases to beat, but even though it stops beating it is well known that it will begin to beat almost immediately after the perfusion is under way. The method as used will be recognized as a slight modification of the old Langendorff method.

The rate and strength of beat were measured by means of a spring lever tied to the tip of the apex. The coronary flow was collected and measured in a tipping bucket which recorded given amounts upon the smoked drum of the kymograph. When the experiment thus described was properly carried out, the rate of beat and coronary flow soon became constant with a liberal flow through the coronary

³ Injection of chloroform, digitoxin and very strong currents applied to the heart stopped it in violent systole and this pressed the dye or ink from the capillaries.

vessels. In the event of faulty solution the coronary flow was very scant and the heart soon ceased to beat. When the constant flow level had been reached, a suspension of Berlin blue, 2 per cent, was injected into the stream near the opening of the coronary arteries. If the injection was successful the heart became almost instantly a dark blue color, and at this point it was stopped by the injection of 5 to 10 cc. of glacial acetic acid or formalin (10 per cent) in alcohol (95 per cent) into the perfusion fluid.

Experiment Cat 3.—February 12, 1926. The cat was killed by a sudden blow upon the head, and the heart immediately excised. A cannula was tied into the aorta and perfusion with Locke-Rosenheim solution immediately started. The perfusion pressure was 45 cm. of water and temperature of the perfusing fluid as it reached the heart was 37°C. The Locke-Rosenheim solution was well oxygenated. Coronary flow was registered by means of the tipping bucket, and after the heart rate and flow became constant a suspension of 2 per cent Berlin blue at a temperature of 37°C. was injected into the perfusing fluid near the coronary arteries, and the heart immediately became a deep blue, showing complete capillary injection. This was followed by 10 per cent formalin in 95 per cent alcohol, which caused the heart to stop beating.

Experiment Rabbit 4.—July 23, 1926. The heart used in this experiment was obtained from a rabbit after a procedure similar to that described in Experiment Cat 3. A cannula was placed in the aorta and perfusion was started with oxygenated Locke-Rosenheim solution at a pressure of 45 cm. of water at 37°C. After the flow became constant and the rate had become constant, Berlin blue was injected, a 2 per cent suspension, into the perfusion fluid. This was followed by glacial acetic acid which stopped the heart immediately. The heart became blue with the exception of a small spot which seemed to have blocked vessels. It was immediately placed in 10 per cent formalin in 95 per cent alcohol for fixation (Figs. 4 and 12).

In view of the complete injection of the capillary bed obtained by this method in the hearts of animals, it was applied to human material obtained at necropsy. Hearts obtained within 3 or 4 hours post mortem began to contract regularly when perfused with oxygenated Locke-Rosenheim solution although in some instances the left ventricle remained inactive if rigor had set in.

Experiment Heart 52.—July 15, 1926. This heart was obtained 2 hours post mortem from a boy aged 15 who died of Hodgkin's disease. In the space of another 20 minutes perfusion was begun with oxygenated Locke-Rosenheim solution at 37°C. and 50 cm. of water pressure. In about 5 minutes after the perfusion was begun the auricles began to twitch, this was followed immediately by regular beats, which in turn were followed by ventricular beats. After 10 minutes or so had elapsed the heart was beating normally, ventricular beats following the auricular

beats in regular order. The rate of beat was 62 per minute. The beats gradually increased in strength and finally the left ventricle was spurting the solution through the aorta with good force. When the flow had become steady a 2 per cent suspension of Berlin blue was injected into the perfusing fluid at such speed as to replace the perfusing fluid.

The heart immediately became a deep blue, but the Berlin blue flow was maintained for about 3 minutes, during which time the heart beat gradually weakened but did not cease. Glacial acetic acid was then run into the perfusing fluid and this stopped the heart instantly.

The heart was then sectioned, each section being carefully measured, after which they were placed in aqueous formalin for fixation (Figs. 5, 6 and 7).

Following such injections, the hearts were sectioned, placed in 10 per cent aqueous formalin or 10 per cent formalin in 95 per cent alcohol, for fixation, and, finally, after the standard preparation, were embedded in celloidin and sectioned. If it was desired to count the number of capillaries per sq. mm., careful measurements were made of the blocks of tissue before and after fixation in order that corrections might be made for the shrinkage during fixation.⁴

Sections of the muscle were stained according to the study to be made. When being prepared for counting capillaries per muscle fibre, a van Gieson or lithium carmine stain (11) was employed. If counts of capillaries per sq. mm. were to be made, the muscle was stained with picric acid, or phloxine for contrast to the color of the injection material.

Method of Counting Capillaries.

The number of capillaries in the heart muscle has been determined in two ways. The total number per sq. mm. of muscle has been counted, and the number of capillaries per 1000 muscle fibres has been counted. In counting the capillaries by either method sections were selected in which the fibres and capillaries were cut transversely. The actual counting was then carried out by placing a ruled micrometer field in the eyepiece of the microscope and making the counts of the capillaries and fibres by the same method that one uses in counting erythrocytes in the ordinary chamber.

In counting the muscle fibres that had been cut in cross-sections slight difficulties were encountered because of the branching structure of the fibres. Thus a fibre sectioned at the exact point of branching sometimes appeared so that it

⁴ All figures in this paper have been corrected for shrinkage. The method for counting capillaries per sq. mm. is adopted in order to compare the number found in normal hearts with that found in pathological hearts. These studies are now in progress.

might have been interpreted either as one or two fibres. Here the personal equation entered in. No claim is made that the counting of the fibres was absolutely accurate, but rules of counting were adopted and followed throughout and the fact that different people were able to make practically duplicate counts was sufficient to show that this error was not a serious one.

In each heart, capillary counts were made from numerous places in the walls of the ventricles, the auricles, the septum and the papillary muscle. The final figures given in the tables represent the average of many counts, and in most instances, the average of the results of several counters. In almost every instance, a thousand or more capillaries were counted in a given area. The exceptions to this, of course, were in making the counts of the auricle, and of the Purkinje fibres where no single area could be found that contained as many as a thousand capillaries cut in cross-section. The counts were made in areas that appeared to be completely injected, and figures from areas where the injection was obviously incomplete are not included.

The results of counts are shown in Tables I to VII. Table I illustrates variations met with in the counts of capillaries from the same heart by different observers. Tables II to IV show the averages of all counts by all observers of the number of capillaries per 1000 muscle fibres. Tables V to VII show the averages of all counts by all observers of the number of capillaries per sq. mm. of heart muscle. The variations in numbers of capillaries in the normal heart of man, cat and rabbit were very slight—so slight indeed that all these hearts can be said to have approximately one capillary for each muscle fibre.

To say finally that these numbers represent a complete filling of every capillary is not possible, but the finding of the same number in various hearts of man, cats and rabbits is strong evidence in favor of a complete filling of all the capillaries. The even distribution moreover, favored total injection for in many instances when injections were incomplete the distribution of capillaries was uneven and the vacant spaces were easily recognized.

In all parts of the heart, with the exceptions of the auricular walls and the Purkinje system, the capillary supply was practically the same. In the auricles, however, the supply was less abundant and the number of capillaries per muscle fibre varied considerably (*cf.* Figs. 5 and 6). In the thickest part of the auricular wall, where the muscle fibres were large, the count approximated in many instances that of the ventricle, but the auricular muscle fibres were usually distinctly

smaller and of less diameter than those of the ventricle. In such areas the number of capillaries per muscle fibre, therefore, was less than in the ventricle. Moreover, in the very thin portion of the auricular wall the number of capillaries per muscle fibre was distinctly less.

TABLE I.
Human Heart 52.

By whom counted	Left ventricle	Right ventricle	Septum	Papillary muscle	Auricle	Purkinje fibres	Muscle immediately adjoining Purkinje fibres
O. A.	1000	1060	1010	1160			
S. W.	828	1196	993	924			
J. T. W.	1091 1108 1046	1133 1100	1040 1018	1079 1083 1058 1076		671 476 595 607 627 631	1040 1018
L. Z.	1004 1050 930 930 930	1080 1120 1010 1040 1030	1090 940 960 930 960	990 940 1009 980 960	511 478 459 484 461 402		
A. C. E.	1072 1067 1067 1060	1054 1069 1072 1094	1083 1074 1032 1065	1104 1101 1077 1061			
Average of all counts	1013	1081	1015	1040	466	601	1029

In comparing quantitatively the circulation of the Purkinje system to that of ordinary heart muscle, it must be remembered that the actual area of the Purkinje system is small and the number of fibres counted consequently was much less than the number of ordinary fibres counted.

TABLE II.

*Human Hearts. Average of All Counts.**Number of Capillaries Per 1000 Muscle Fibres.*

Human No.	Left ventricle	Right ventricle	Septum	Papillary muscle	Auricle	Purkinje fibres	Muscle immedi- ately adjoining Purkinje fibres
40	1080	1030	950		550		
		1120	1080		520		
		1110	1110		510		
		1110			570		
		1060					
		1123					
		1126					
		1052					
		1070					
		1160					
		1080					
		1100					
		1000					
52	1000	1060	1010	1160	511	671	1040
	828	1196	993	924	478	476	1018
	1091	1133	1040	1079	459	595	
	1108	1100	1018	1083	484	607	
	1046	1080	1090	1058	461	631	
	1004	1120	940	1076	402	627	
	1050	1010	960	990			
	930	1040	930	940			
	930	1030	960	1009			
	930	1054	1083	980			
	1072	1069	1074	960			
	1067	1072	1032	1104			
	1067	1094	1065	1101			
	1060			1077			
				1061			
57	1058	965	1092	1090	608		
	1170	985	1131	1030	567		
	1214	971	1090	1080	541		
	1177	941	1046	1080	505		
	1183	940	1068	1080	441		
	1165	978	1082	1084	460		
	1070	946	1071	1108			
	1166	970	1038	1040			
	1060		1034	1042			
	1120		1109	1130			

TABLE II—*Concluded.*

Human No.	Left ventricle	Right ventricle	Septum	Papillary muscle	Auricle	Purkinje fibres	Muscle immediately adjoining Purkinje fibres
58	1024	1200	1236	1060	493		
	1104	1080	1180	1080	546		
	1060	1200	1143	1090	508		
	1159	1146	1110	1050	513		
	1135	1081	1095	1080	500		
	1242	1133	1087	1069	482		
	1085	1140	1099	1094			
	1095	1088	1083	1110			
	1233	1072	1139	1080			
	1145	1087	1127	1156			
Average of all counts	1075	1032	1071	1060	503	601	1029

The main branches of the Purkinje system lie immediately beneath the endocardium, and the sections in which the capillaries were quantitated were from the papillary muscles or from the septum. The Best stain for glycogen was first used to identify the conducting system but the hemotoxylin so obscured the blue dye in the capillaries that it was impossible to count them. Consequently the Best stain was modified by Miss Louise J. Zschiesche⁵ so that when tissues had

⁵ The method was carried out in the following manner:

1. Stain the sections lightly with alum-hematoxylin for 10 seconds or longer, enough to bring out the nuclei.

2. Remove and wash in several changes of water.

3. Stain for 15 to 20 minutes in the following solution as found in Mallory and Wright (Mallory, F. B., and Wright, J. H., *Pathological technique*, Philadelphia, 8th edition, 1924, 199).

Stock carmine solution freshly filtered..... 2 cc.

Household ammonia..... 3 cc.

Methyl alcohol 95 per cent..... 3 cc.

Filter.

4. Differentiate for 5 to 7 minutes in the following solution, changing the fluid two or three times until it remains uncolored:

Absolute alcohol..... 80 cc.

Methyl alcohol 95 per cent..... 40 cc.

Distilled water..... 100 cc.

been through the process the capillaries remained clearly visible. This stain showed the glycogen as sharply as the Best stain.

The capillary supply to the Purkinje system was found to be much less than that to the heart muscle and the supply decreased rapidly as the endocardium was approached (see Figs. 13 and 8). Thus the Purkinje fibres lying immediately beneath the endocardium had an average of about 600 capillaries per 1000 fibres, while the muscle fibres lying more centrally in a papillary muscle averaged in two counts, 1029 capillaries per 1000 fibres. This is shown beautifully in Fig. 13. In some instances very small clumps of Purkinje fibres, made up of ten or twelve fibres, had a blood supply equal to that of the muscle—one capillary per fibre—but this finding was an unusual one.

When the entire capillary bed was completely injected, the enormous wealth of the heart in capillaries came to light. Practically every heart fibre was in direct contact with one capillary while many were touched by two or more capillaries. The anastomoses between the capillaries were so numerous indeed that these branchings running across the parallel muscle fibres wove a tangled structure of muscle and blood vessels so complex that one fibre was often completely surrounded by capillaries. The longitudinal sections showed this interrelated mesh of fibres and capillaries very strikingly (Figs. 3 and 4).

When the tissue was fixed and embedded in such a way that the shrinkage caused the fibres to stand out separately it was clear that the capillaries lay between and did not actually pierce the muscle fibres, but their intimate entwining about the fibres and through the forks of branching heart fibres frequently gave the appearance of their actually entering the substance of a fibre.

-
5. Wash in several changes of 80 per cent alcohol.
 6. Wash in one change of 95 per cent alcohol.
 7. Stain about 30 seconds in 95 per cent alcohol to which a saturated solution of picric acid has been added (5 cc. picric acid solution; 50 cc. 95 per cent alcohol).
The length of time the sections are left in the picric acid and alcohol depends on the intensity of the color desired for the cytoplasm.
 8. Wash in several changes of 95 per cent alcohol.
 9. Oil origanum.
 10. Balsam.

The long standing controversy over the occurrence of blood vessels in the heart valves has received renewed attention in the past few

TABLE III.

*Cat Hearts. Average of All Counts.
Number of Capillaries Per 1000 Muscle Fibres.*

Cat No.	Left ventricle	Right ventricle	Septum	Papillary muscle	Auricle	Purkinje fibres
3	1080	1048	1070	1043	701	
	1035	1085	1070	1029	490	
	1006	1068	1008	1024	458	
	1089	1072	1000	1027	463	
	1058	1035	1042	1056	486	
	1085	1102	1057	1030	650	
	1114	1046	1053	1033	558	
	1074	1058	1056	1044	509	
	1094	1050	1066	1046		
	1129		1051	1043		
4	1050	1002	1100	897	370	
	976	1100	1037	970	490	
	1002	833	920	1008	423	
	989	979	942	990	402	
	1023	940	971	1000	455	
	1021	970	910	1061	358	
	1005	979	964	956	485	
	1016	981	1000	957	462	
	978	947	971	1014	454	
	971					
5	1071	1073	1079	1084	608	638
	1021	1087	1115	1110	567	
	1063	1071	1086	1102	492	
	1073	1048	1026	1096	686	
	1063	1051	1064	1018	374	
	1050	1048	1058	1125	405	
	1106	1076	1047	1048	456	
	1058	1069				
	1061					
Average of all counts	1046	1031	1029	1031	491	638

years and several contributions have been made. Numerous methods have been employed to inject the vessels of the valves and the results

TABLE IV.

*Rabbit Hearts. Average of All Counts.
Number of Capillaries Per 1000 Muscle Fibres.*

Rabbit No.	Left ventricle	Right ventricle	Septum	Papillary muscle	Auricle
1	1140	1143	1118	1073	675
	1114	1131	1140	1084	655
	1070	1150	1030	1030	528
	1080	1200	1082	1030	521
	1030	1122	1100	1040	461
	1100	1040	1150	1030	542
	1140	1219	1102	1235	604
	1033	1117	1117	1096	574
	1121	1084	1122	1103	649
	1080	1091	1108	1104	623
	1081	1107		1108	
	1029				
	1126				
2	1128	1103	1160	1107	634
	1020	1100	1099	1057	730
	1038	1100	1047	1087	666
	1086	1129	1038	1080	747
	1070	1060	1121	1017	581
	1094	1050	1118	1170	463
	1107	1070	1093	1093	761
	1070	1000	1070	1032	568
	1025	1010	1086	1047	747
	1097	990	1090	1000	
		1128			
3	1027	1130	1049	1127	528
	1021	1084	1024	1220	604
	1066	1192	1048	1023	408
	1024	1081	1020	1044	429
	1021	1066	1017	1072	407
	1106	1020	1064	1008	403
	1021	1061	1046	1046	
	1106	1054	1075	1020	
	1021	1049	1068	1065	
	1069	1064	1120	1008	
	1035				
	1036				

TABLE IV—*Concluded.*

Rabbit No.	Left ventricle	Right ventricle	Septum	Papillary muscle	Auricle
4	1130	1093	1082	1150	666
	1098	1061	1077	1031	436
	1010	1009	1092	1028	385
	1070	1051	1038	1043	394
	1080	1050	1056	1063	463
	1041	1014	1070	1049	413
	1019	1029	1092	1024	511
	1028	1019	1060	1009	
		1030	1031	1016	
			1100		
5	1223	1039	1224	1135	597
	1100	1057	1037	1104	414
	1095	1064	1044	1050	506
	1122	1041	1044	1091	590
	1087	1030	1081	1090	511
	1047	1043	1053	1070	477
	1038	1020	1068	1008	
	1022	1035	1038	1022	
	1060	1019	1059	1046	
	1048	1054	1105	1041	
Average of all counts	1071	1072	1075	1044	520

TABLE V.

*Human Hearts. Average of All Counts.**Capillaries Per Sq. Mm.*

Human No.	Left ventricle	Right ventricle	Septum	Papillary muscle	Purkinje fibres	Muscle immediately adjoining Purkinje fibres
52	5852	5330	4480	4600	2360	4920
	5674	5824	4427	4960	1800	5400
	5720	6023		5600	1524	
	5808	5416		4640	2176	
	5674	5590		5464	2840	
	5632	5893		5240	2880	
	5776			5600		
				5360		
				5600		
				4920		
				5400		
Average of all counts	5734	5679	4453	5216	2263	5160

TABLE VI.

Cat Hearts. Average of All Sq. Mm. Counts.

Cat No.	Left ventricle	Right ventricle	Septum	Papillary muscle	Auricle	Purkinje fibres	Muscle immediately adjoining Purkinje fibres
5	3400	4668	4676	4800	3204	1802	4340
	5040	4015	3181	3240	3131		
	3040	4316	3040	3080			
	4408	4216	4480	4436			
	4424	3857	4340	4340			
	4224	3997					
Average of all counts	4089	4178	3943	3979	3167	1802	4340

TABLE VII.

Rabbit Hearts. Average of All Sq. Mm. Counts.

Rabbit No.	Left ventricle	Right ventricle	Septum	Papillary muscle
3	6336	5676	4160	5413
	6292	5806	4096	5600
	6248	5763	4000	6393
	5896	5720	4000	5413
	5764	6196	4000	7000
	5896	6673	4064	5693
	5852	6933	4064	5413
4	5720	6504	7100	5600
	5490	6461	6480	6461
	5320	6030	6800	5240
	5120	5772	6100	6480
	5160	6076	6300	5480
	5400	5987	6600	5680
	4960	5815	7150	5400
				5680
				5960
Average of all counts	5674	6101	5351	5806

obtained have been very variable. No one has found the vessels constantly present in all valves and this is about the only point upon which all workers seem to agree.

Bayne-Jones (12), Kugel and Gross (13) and Kerr and Mettier (9) have helped to bring order from the mass of contradictory evidence, by demonstrating clearly the presence of vessels in many valves. The difference in the methods of injection used in the past probably accounts for the conflicting claims made. Kugel and Gross have used a method which injects only the finer arterioles as they were not interested in the capillaries. These workers have found the vessels to be inconstant but present in many valves. Kerr and Mettier, on the other hand, have made beautiful injections of the capillaries in the heart valves of pigs.

Before any final verdict can be rendered upon the subject a sufficiently large number of hearts must be studied in order to establish the average occurrence. By injecting the beating heart it has been possible to demonstrate vessels in the auriculoventricular valves in many instances, and in the semilunar valves in a few instances, and the distribution of the vessels showed the injection to be complete (Fig. 9). Instead of ending blindly in the valve the capillaries could be seen to go down onto the leaflet of the valve, make an unbroken loop and return to anastomose with the vessels at the base of the valve. Similarly the vessels in the papillary muscles ran up to the chordæ tendineæ, looped over and returned toward the base of the muscle. In rare instances the vessels ran through the chordæ tendineæ to anastomose with the capillaries on the free portion of the valve (Fig. 10).

It was also true, on the other hand, that in other perfectly injected hearts in which the muscle capillaries and vessels at the bases of the valves were completely filled, the valves showed no vascularity. These valves when cleared showed beautifully injected intact capillary loops at their bases, but none of the capillaries ran down upon the valve proper.

In view of the fact that the first part of the aorta receives a blood supply from a branch of the coronary artery, it was not surprising to find its wall well filled with capillaries (Fig. 11). The parallel course of the arteries and veins in the aortic wall is very striking and characteristic of the vasa vasorum in the wall of this vessel. This rich blood supply in the general region where aortitis is most common may be of important clinical significance.

SUMMARY.

By means of injections made into the coronary arteries of beating hearts it has been possible to determine the number of capillaries in the normal heart muscle. This study has shown a very rich blood supply with an average of approximately one capillary for each muscle fibre in the ventricular walls and papillary muscles, and a less abundant supply in the auricular muscle and Purkinje system. The number of capillaries per sq. mm. of ventricular wall or papillary muscle is about twice that found by Krogh in skeletal muscle. Capillaries were not found constantly in the valves of hearts in which there was apparently a complete injection of the capillary bed. The method described for injecting the capillaries of the heart also provides a means of studying the blood supply to the muscle, valves and aortic wall in pathological hearts.

We wish to express our appreciation to Misses Sylvia Warren and Olivia Ames, and to Dr. A. C. Ernstene for their assistance in making many of the capillary counts shown in the tables.

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EXPLANATION OF PLATES.

PLATE 13.

FIG. 1. 500 diameters. Human Heart 57. Ventricle. Good injections of capillaries made 48 hours post mortem. The injection was made through the veins without previous washing out of the vessels.

FIG. 2. 500 diameters. Human Heart 57. Ventricle. An incompletely injected area from a point near that shown in Fig. 1.

FIG. 3. 700 diameters. Cat 2. Ventricle. Longitudinal section of capillaries showing numerous anastomoses.

FIG. 4. 500 diameters. Rabbit 4. Papillary muscle. Cross-section of capillaries showing a field similar to that in Fig. 12.

FIG. 5. 500 diameters. Human Heart 52. Shows irregular and less abundant circulation of the auricle. Compare with Fig. 6.

PLATE 14.

FIG. 6. 500 diameters. Human Heart 52. Longitudinal section of ventricle showing several anastomoses of capillaries.

FIG. 7. 500 diameters. Human Heart 52. Ventricle showing cross-section of muscle fibres and capillaries.

FIG. 8. Approximately 150 diameters. Human Heart 52. Papillary muscle showing Purkinje fibres at the upper edge of the section. Note the poorer supply of capillaries in the Purkinje tissue when contrasted with the adjoining muscle.

PLATE 15.

FIG. 9. Human Heart 37. Cleared mitral valve, aortic cusp, showing clumps of capillaries in the valve. Note that the capillaries do not end blindly, but anastomose by loops with others nearby.

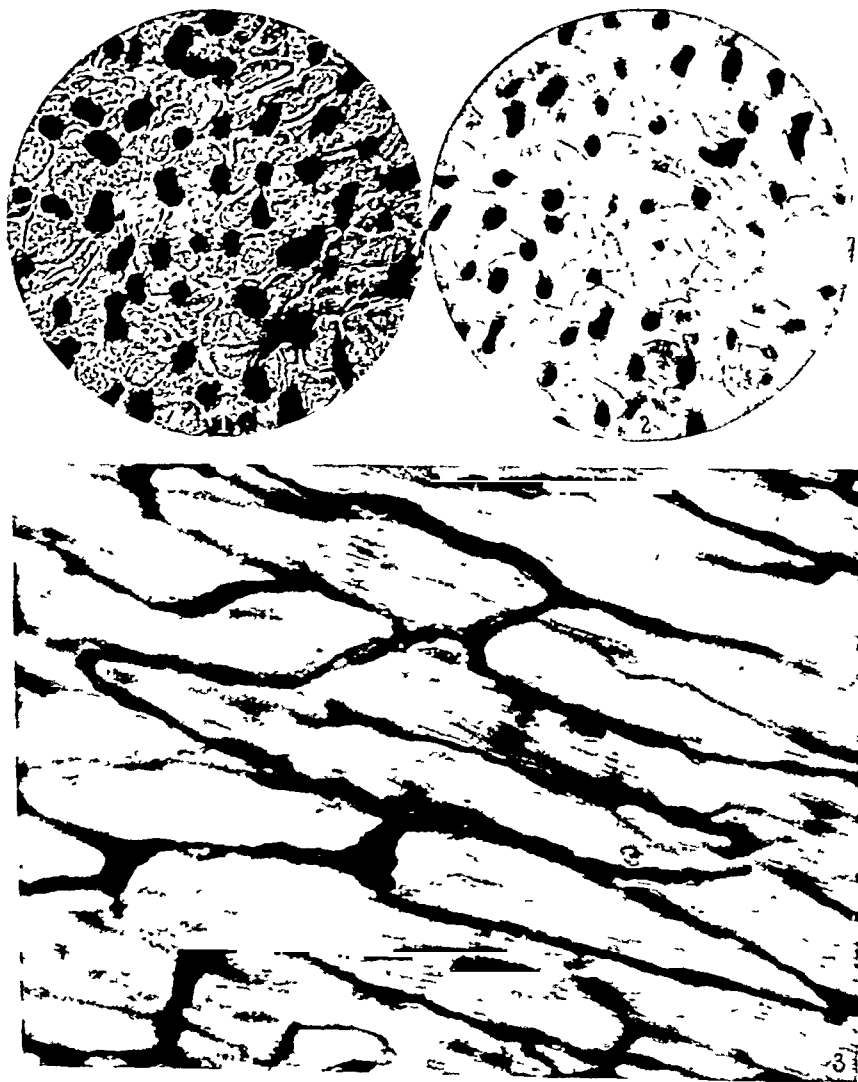
FIG. 10. Human Heart 37. Mitral valve showing anastomoses of vessels in chorda tendinea at the lower part of the plate with vessels on the free portion of the valve.

FIG. 11. Human Heart 52. Cleared section of aortic wall 1 cm. from the aortic valves.

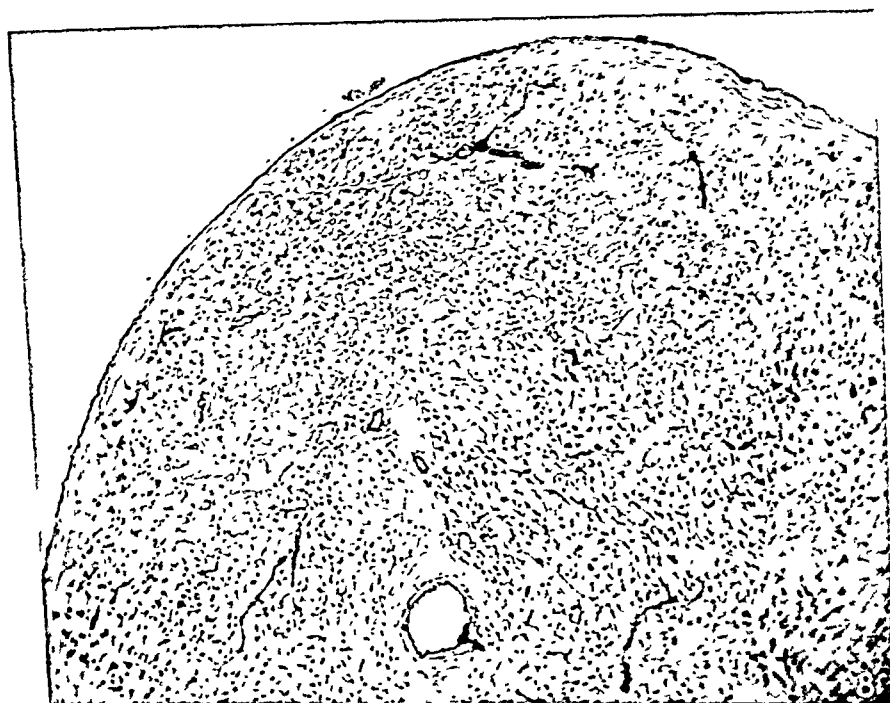
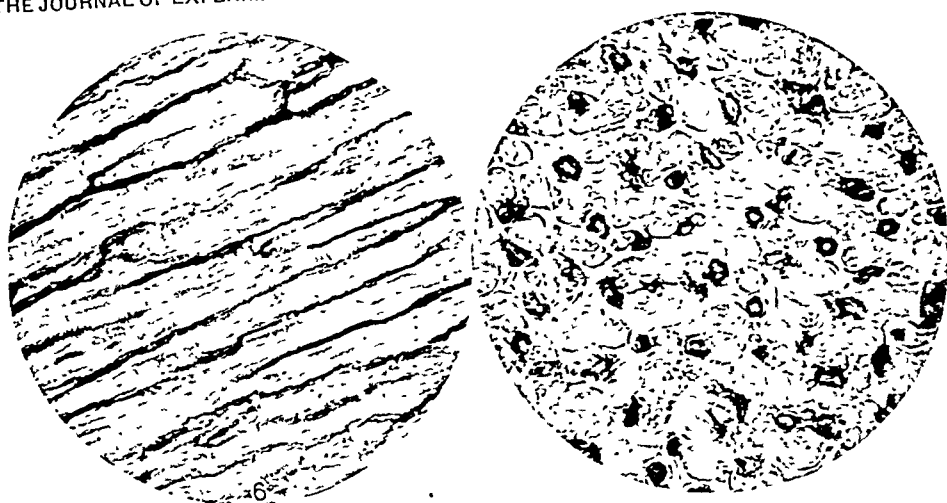
PLATE 16.

FIG. 12. Ventricle of Rabbit 4. Lithium carmine stain, showing red nuclei and red muscle fibres. The capillaries are filled with Berlin blue. This stain was employed to differentiate capillaries and cell nuclei, in the sections in which the capillaries were counted. This prevented confusion of nuclei and capillaries, and differentiated more clearly the single muscle fibre.

FIG. 13. Human Heart 52. Papillary muscle. Zschiesche modification of Best's stain. Capillaries are blue, Purkinje fibres are red and the ordinary heart muscle fibres are yellow. This plate shows much more clearly than Fig. 8 the difference in blood supply to the heart muscle and the Purkinje system.



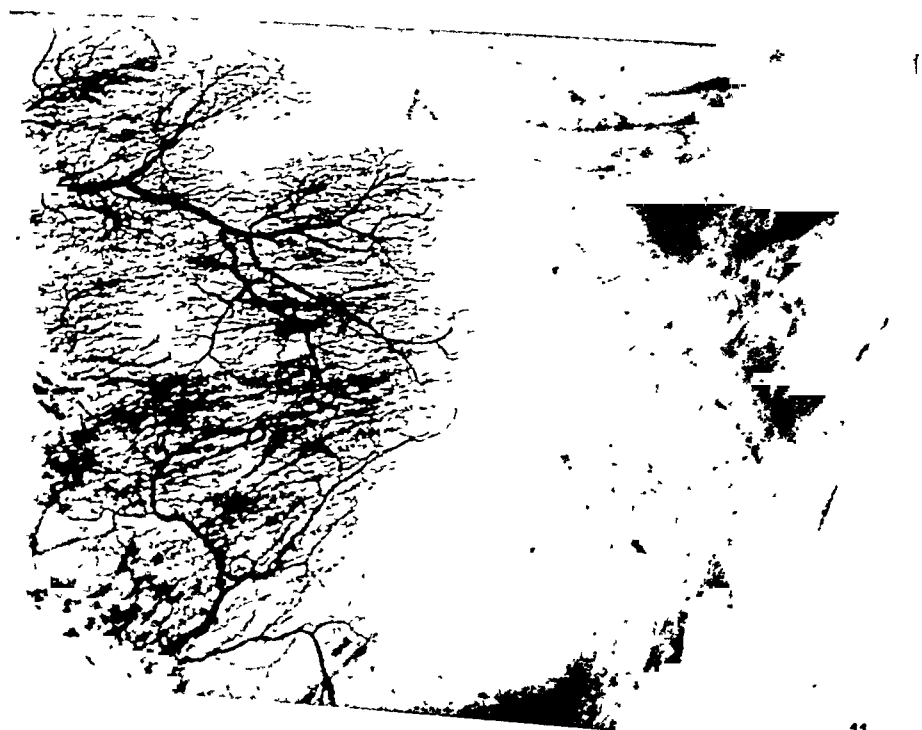
(Wern. Capillary bed of heart.)



(Wearn: Capillary bed of heart.)

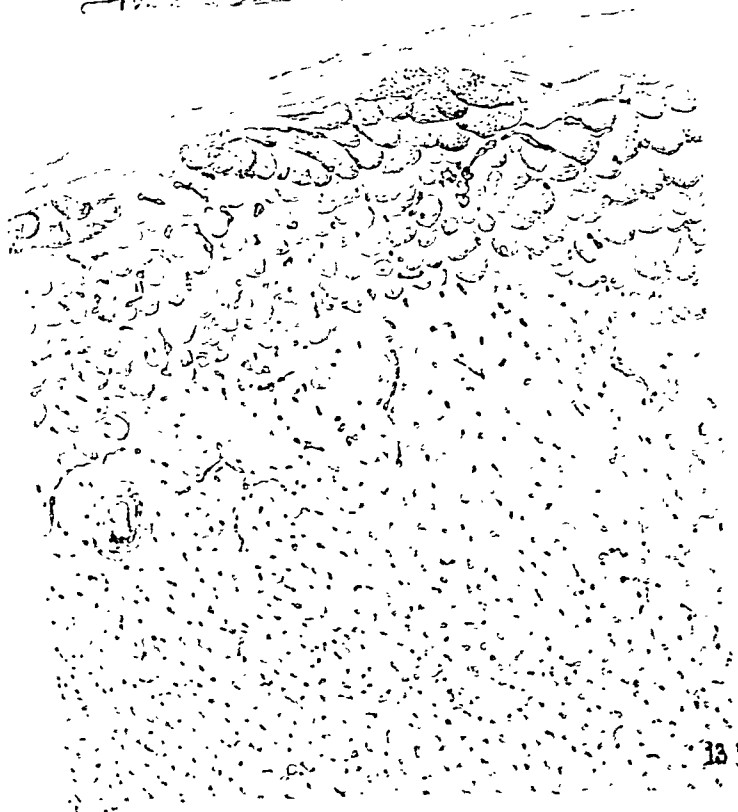
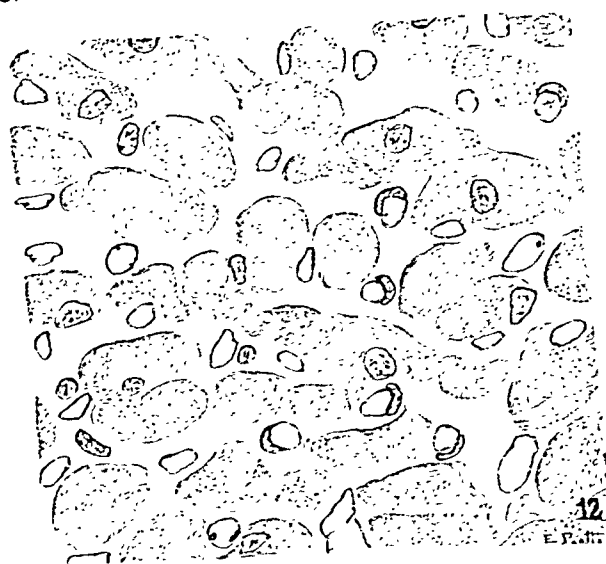


9



11

(Wearn: Capillary bed of heart.)



THE RÔLE OF THE THEBESIAN VESSELS IN THE CIRCULATION OF THE HEART.

By JOSEPH T. WEARN, M.D.

(From the Thorndike Memorial Laboratory of the Boston City Hospital, and the Department of Medicine, Harvard Medical School, Boston.)

PLATES 17 AND 18.

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Interest in the coronary circulation and blood vessels of the heart was very keen among the anatomists and physicians in the early part of the 18th century. They had begun to study the distribution of the coronary vessels and as a result of their dissections to construct theories of their function. Perfusion experiments were also carried out on human hearts obtained at necropsies and rapid advances were made in the knowledge of the coronary circulation. Text-books and anatomical works of the day devoted much space to the subject and clinicians began to correlate the postmortem with the clinical findings. The belief that special substances other than blood circulated in the blood vessels had begun to disappear.

This interest in the circulation of the heart walls was given a fresh stimulus by the publications of Raymond Vieussens in 1706 and by those of Adam Christian Thebesius 2 years later. Their discoveries of the channels connecting the coronary vessels directly with the chambers of the auricles and ventricles, introduced into the scheme of the circulation of the heart the vessels which are now generally known as Thebesian veins. In more recent times these vessels have received very little attention or study. Indeed, little is known of their anatomical relationships and no exact knowledge of the part they play in the coronary circulation exists. The importance, however, of a group of vessels which plays a part in the circulation of the heart itself, is obvious. It is the purpose of this paper to describe the previous work on the Thebesian vessels and their supposed functions and in addition, to present some observations made during the past 3 years

upon the relationship of these veins to the coronary arteries and upon some of their functions.

LITERATURE.

In 1706 Raymond Vieussens (1) published the results of a series of experiments which he had carried out upon human hearts after death together with others on beef and sheep hearts. In these experiments Vieussens ligated the vena cava above and below the right auricle of the heart and next ligated the pulmonary veins. Having thus blocked these outlets he injected a solution of safranine in alcohol into the coronary arteries. After the safranine had filled the tissues of the heart, he observed that it ran out, not only through the coronary sinus into the right auricle, but—and this is the point of the experiment—it also escaped directly into the cavities of the heart by way of small ducts in the walls of the auricles and ventricles. These openings he termed “ducti carnosī.” He found a considerable number of them on the interior surfaces of the heart and after an extensive study concluded that they were continuous with the coronary arteries.

In 1708 Thebesius (2) published his description of the numerous openings for the venous blood in the auricles and ventricles. He identified the openings by injecting the water into the coronary sinus and observing its escape into the chambers. The same results were obtained by injecting air into the coronary veins, and on still other occasions he employed colored liquids mixed with wax and glue. Thebesius raised the question as to why the Creator had placed these small veins in the walls of the heart, and then very obligingly answered it by saying that they made possible a continuous blood flow in the heart by serving as an exit at the beginning of systole. After thus answering the question so satisfactorily to himself, he gave due credit and much praise to the Creator for being so foresighted as to anticipate the need and usefulness of these little vessels. Thebesius also stated that while he was writing his paper he was shown the report of Vieussens' experiments. Despite the fact, therefore, that the vessels bear the name of Thebesius, it is to Raymond Vieussens that credit for their discovery should go. He reported his findings first, but of even greater importance was his method of injection, namely, through the arteries. Thebesius injected the veins and both men described the same openings.

Philip Verheyen (3) in 1712 reviewed the work of Vieussens and repeated some of his experiments. He conceded that the experiments were very unusual, but added that Vieussens, like other great men, had drawn too many conclusions from them. Verheyen confirmed the presence of the openings in the walls of the heart cavities, but explained them as openings of coronary veins and denied rather emphatically that they were special ducts. Neither his experiments nor his arguments in favor of his claims were very convincing.

In 1715 Vieussens published a book (4) in which he confirmed his former discovery of the common openings and spoke of their distribution and function. It was in this delightful publication that he described in detail stenosis of the mitral

valve and the clinical and necropsy findings in a patient with insufficiency of the aortic valve.

Lancisi (5) repeated the experiments and confirmed the results of Thebesius. His explanation of the *raison d'être* of the Thebesian system is interesting even if not based upon experiment. Assuming that the heart beat was started by the rush of blood into the heart muscle, he thought a rapid and simultaneous emptying necessary in all parts of the heart. In the parts most distant from the right auricle the necessary exits were supplied by the Thebesian vessels, and by this means, he said all parts could be emptied simultaneously and as a result all parts could be filled instantly.

Sénac is quoted (6) as saying that injections of air, mercury or tallow pass into the ventricles of the heart directly and enter these cavities equally well whether poured into the coronary artery or coronary vein. Langer (7), Bochdalek (8) and many others have admitted the presence of these vessels and studied their distribution in the walls of the four chambers. Nussbaum (9) also studied the numbers and sites of the openings but concluded that they existed only in the auricles.

On the other side of the question, however, are arrayed Cruveilhier (10) and Theile (11) who denied that such vessels existed. In reading their views one is led to believe that they are opinions only, and not based upon original observation. Lannelongue (12) too, denied that the small openings in the heart walls were connected with the arteries and veins. He maintained that they were merely continuations of the numerous small canals that ran behind the plexus of pillars in the walls of the ventricles.

Haller has been quoted by several writers as denying the existence of Thebesian vessels but in one of his writings (13) he spoke of their presence and stated that their function was to return the blood of those deep seated coronary arteries which were unaccompanied by veins.

In more recent years Pratt (14) has demonstrated that an isolated cat heart can be kept beating for an hour by the perfusion of defibrinated blood through the Thebesian vessels. He believed the Thebesian veins were connected directly with the coronary veins and indirectly with the arteries through the capillaries. Pratt, moreover, explained the lack of infarcts in markedly sclerosed hearts by the presence of the Thebesian circulation.

The embryological development of the Thebesian veins has not been studied exhaustively but the few papers that exist indicate that these vessels are the remains of the primitive intertrabecular circulation of the embryo. Minot (15) found that the trabeculae of the heart were made up in their earliest stage of young heart muscle cells covered with endothelium, but without capillaries of their own. It was his belief that the "sinusoidal" circulation of the embryonic heart was sufficient to nourish the trabecular muscle. Lewis (16) confirmed the work of Minot and followed the development of the sinusoids of the heart in the torpedo and rabbit and believed them to be the vessels that survive to some extent in the adult hearts, as the Thebesian veins.

Grant (17) has found that in the beginning the sinusoids were not connected with the coronary system, but grew into them at a later stage, as the coronary veins grew and branched in the myocardium. He (18) also described an anomaly of a child's heart in the ventricular muscle of which were large blood-filled spaces, communicating freely with the cavity of the ventricle as well as with the coronary vessels. They were interpreted as a persistence of the sinusoidal spaces of the embryonic heart.

In the hearts of certain fishes, turtles and frogs, the sinusoids persist in adult life, and it is through them that the inner surface of the heart wall is nourished. No capillaries have been found in the spongy surface supplied by the intertrabecular circulation.

Methods.

The observations which stimulated the work to be reported upon in this paper, were made during attempts to inject the capillaries of a

TABLE I.
Human Heart 17.

	1st measurement	2nd measurement
	cc.	cc.
Outflow from pulmonary artery.....	70	73
Outflow from aorta.....	190	190
Outflow from coronary sinus and veins.....	28	26
Leaks from cut edges of heart.....	60	52

human heart. The method which was employed for the injection was as follows:

About 48 hours after death a human heart was washed free of blood by perfusing salt solution (0.85 per cent) through the coronary arteries at a pressure of 160 mm. Hg. When the perfusate began to escape it was noted that the greater part of it ran out of the aorta and pulmonary artery and very little flowed from the coronary sinus. This observation was so startling that steps were taken immediately to measure the outflow from heart cavities and from the coronary sinus and other great veins. Glass cannulæ were introduced into the aorta and pulmonary artery in such manner that their ends extended well into the chambers of the ventricles. The coronary sinus and the posterior middle vein were also cannulated. At times several of the great veins emptied directly into the right auricle near the opening of the coronary sinus and just outside the valve of Vieussens. Whenever possible these veins were cannulated and the flow from them was

recorded. The coronary arteries were then perfused for 1 minute with normal salt solution at a pressure of 160 mm. Hg, and the perfusate was collected as it escaped from the various sources, and measured.

The results of such an experiment are shown in Table I.

Perfusion of salt solution (0.85 per cent) through the coronary arteries for 1 minute at 160 mm. Hg pressure.

Such results as those shown in Table I stimulated further study, and the experiment was repeated with the hearts obtained from a number of necropsies. Some of these were normal hearts, others were hypertrophied, while still others showed marked sclerosis of their vessels. The results of these experiments confirmed the findings in the earlier one (see Table II) namely, that 60-90 per cent of the coronary flow escaped through the Thebesian vessels directly into the chambers of the heart.

The possibility of rupture of some of the larger coronary vessels directly into the chambers or transudation of the salt solution into the cavities from the vessels was thought of as an explanation of the small amount of flow from the veins. Direct inspection, however, of the inner walls of the heart during perfusion with India ink showed the ink escaping only from the Thebesian vessels. Moreover, when the hearts were sectioned later, careful search failed to reveal any extravasation of the ink whatsoever. Further confirmation of the Thebesian vessels as the only source of the outflow from the chambers was furnished by perfusing with a suspension of gum acacia (6 per cent) in salt solution. This substance behaved in a manner similar to the saline perfusion, except that, as was to be expected, it ran more slowly (see Table II, Heart 43).

In several experiments agar and celloidin colored with dyes were perfused through the coronary arteries, and during the perfusion cold water or ice was introduced into the ventricles to harden these substances *in situ*. Subsequent sectioning of these hearts revealed small plugs of the injection mass protruding from the Thebesian openings. In one of the hearts in which celloidin was used as the injection mass seventeen openings of Thebesian vessels were found in the left ventricle with plugs of celloidin protruding from them (Fig. 1). The injection pressure used was 220 mm. of mercury and the mass was so thick

TABLE II.

Human heart No.	Age of patient yrs.	Necropsy diagnosis	Weight of heart gm.	Perfusate used	Flow from left ventricle (Thebesian veins) cc.	Flow from right ventricle (Thebesian veins) cc.	Flow (Thebesian veins) percent	Flow from coronary sinus and vein cc.	Leaks from cut surfaces cc.	Pressure of perfusate mm. Hg	Amount of flow	Condition of coronary arteries
15	45	General peritonitis	320	Physiological salt solution .85 per cent	88 79	122 120	42 44	105 100	175 151	190	490 450	Normal
16	45	Chronic nephritis	500	Potassium ferrocyanide 3.2 per cent	75 70	125 125	76 73	20 27	40 45	155	260 267	Moderate arteriosclerosis
17	50	Acute purulent meningitis?	410	Physiological salt solution .85 per cent	176 190 190	65 70 73	73 76 91	25 28 26	60 52	160	326 340 289	" "
20	50	Sarcomatosis	325	" "	235 240	250 260	78 74	50 80	80 95	220	615 675	" "
21	60	Chronic myocarditis; heart failure	300	" "	100 100	142 130	74 70	37 45	47 53	180	326 328	Slight sclerosis of arteries
22	51	Chronic nephritis	400	" "	175 175	235 215	74 74	20 15	120 120	150	550 525	Moderate sclerosis
24	42	Bronchopneumonia	220	" "	180 185	125 122	69 70	26 31	105 100	180	436 438	Normal

25	50	"	260	"	0	60	68	4	23	180	87 91	Moderate arterio- sclerosis
26	65	Pulmonary tuber- culosis	500	"	1	62	69	5	23	150	453 462	"
27	not 19	Bronchopneu- monia		"	183 176	200 208	84 83	10 8	60 70	150		Normal
37	not 40	"		"	19	32	34	16	80	150	147	Arteriosclerosis
43	43	Pulmonary tuber- culosis	270	"	0	100	23	30	300	150	430 433	Normal
				"	0	100	23	33	300	150		Arteriosclerosis
				"	140 152 175 168	215 210 175 163	72 72 58 59	60 64 75 65	75 70 170 160	150	490 496 595 556	Normal
				Acacia 6 per cent								
				Physiological salt solution .85 per cent	232 246		62 64	56 60	85 75	150	373 381	Arteriosclerosis
46	65	Cerebral hemor- rhage	510	"	210 200	300 289	76 76	35 30	125 120	150	670 639	"
47	82	Chronic nephritis myocarditis	580	"								

These experiments were carried out as nearly as possible under exactly the same conditions. All injections were made approximately 48 hours post mortem. The temperature of the perfusate was kept at 37°C. and with the exception of Heart 43 all of the outflows were collected during 1 minute periods. In Heart 43 the salt solution was perfused for 2 minutes and the acacia for 5 minutes.

that it did not enter the capillaries. Indeed, only the larger vessels were injected. No openings were found in the auricles and only two were found in the right ventricle. These experiments, therefore, confirm the previous ones (Table II) for here again the capillaries were not filled. Finally microscopic examination of sections from numerous parts of the hearts showed that the dyes and injection material had remained within the walls of the vessels.

Crainicianu (19), in perfusing hearts, noted this large escape from the ventricles but did not pursue it further. In 1913 Starling and Evans (20) during a study of the metabolism of the heart and lungs noted that about 40 per cent of the coronary blood did not return *via* the coronary sinus. They did not state, however, whether they took into consideration the other great veins which empty directly into the right auricle or the Thebesian flow to the left auricle and ventricle.

On gross inspection of the hearts used in the experiments of Table II it was obvious that the injection was uneven and incomplete. The larger vessels were well filled with ink or dye, but for the most part the heart was not colored by the injection material. Sections from numerous points in the walls of all the chambers were prepared and a careful microscopic study of these brought out the surprising fact that in most instances scarcely any of the capillaries were injected. The large arteries and veins were everywhere well filled or stained with the injection mass, but in the great majority of the microscopic fields very few if any capillaries could be found (Fig. 2). In certain fields small groups of capillaries were filled, but in no instance in the study of fifty human hearts was the capillary bed entirely injected. Even in the hearts in which some of the capillaries were injected they were so few in number that it was obviously impossible for them to take care of the profuse flow from the arteries into the cavities of the auricles and ventricles. In two hearts there was a scant flow through the Thebesian vessels, and in these several areas showed almost completely filled capillaries. The injection, however, was uneven and several large spots in the walls remained uninjected.

Here then is evidence that a direct connection exists between the coronary arteries and the chambers of the heart. The fact that so great a flow between the two can take place without going through the

capillaries shows the connecting channels to be of considerable size. The nature of this connection has not been definitely determined. It may possibly be a direct one from artery to Thebesian vessel, or more probably it may go from an artery into a large vein and thence into the heart cavity. Two of the smaller Thebesian vessels have been traced by serial sections, and their branchings studied. For convenience the smaller papillary muscles were selected because in so small a structure the course of the vessel would necessarily be a short one. In the two vessels so studied large venous branches were given off immediately beneath the endocardium and these branches divided immediately into venules and capillaries as illustrated in Fig. 3. Some of the Thebesian veins, therefore, drain the capillaries. This outlet for the capillary blood obviates the longer route of return through the veins to the coronary sinus and right auricle. The study has not progressed far enough to determine the relative number of Thebesian veins that drain capillaries as compared to those that connect directly with larger veins or through them with arteries. It is clear, however, that three types of connections exist, (1) the direct connection between the arteries and the Thebesian vessels, as shown by the celloidin injections, (2) the venous connection with the Thebesian vessels and (3) the capillaries which run directly into the Thebesian vessels.

The distribution of the Thebesian openings varied greatly but they were usually most numerous in the walls of the ventricles, in the pocket of the apex and in and around the bases of the papillary muscles. In the hearts studied there were relatively few openings into the auricles, and in two instances it was not possible to demonstrate any openings at all in either auricle. This was determined by measuring the flow from each chamber separately. The following protocol is illustrative.

Protocol.

Heart 47.—Male. Age 82. Chronic myocarditis with heart failure. A human heart, 24 hours post mortem, had cannulae placed in the pulmonary artery, the coronary sinus and posterior middle vein, in the aorta and in the coronary arteries. The mitral valve was sewed together very tightly, as was also the tricuspid valve, in such fashion that they were water tight. A cannula was then sewed into each auricle. Following this, the coronary arteries were perfused at a pressure of 150

mm. of mercury, and flows from the various chambers were recorded for 1 minute periods. They were as follows:

	1st measurement	2nd measurement
	cc.	cc.
Pulmonary artery.....	300	289
Aorta.....	210	200
Coronary sinus and vein.....	35	30
Right auricle.....	0	0
Left auricle.....	0	0
Leaks.....	125	120

Following these measurements Berlin blue was injected into the coronary arteries at a pressure of 150 mm., and simultaneously carmine solution was injected through the coronary sinus and the posterior middle vein. The injection pressure continued for a few minutes following which the heart was opened and examined. It was interesting to note that most of the blue had emerged near the apex in each ventricle. The red was not so well localized. Neither red nor blue appeared in the auricles.

The number and size of Thebesian vessels in the various chambers also varied greatly. At times the flow was much greater from the right side while at others greater from the left, but the average from all the hearts showed a slightly greater flow from the right. The difference, however, was not significant.

Changing the perfusion pressure did not change the relative proportion of flow from the two sides of the heart, but did affect the rate of flow. Nor did the temperature of the heart influence the flow, for perfusions were carried out at various temperatures without effect upon the amount. It might be stated, however, that with few exceptions all the hearts were kept from 24-48 hours in the ice box, and then gently massaged and brought to a temperature of 45°C. before being perfused.

Attempts to inject the capillaries of a heart while in rigor mortis met with no better success. Nor did the use of this method with fresh warm hearts obtained soon after death give any better results. In still other hearts potassium sulfocyanate (10 per cent) was perfused through the vessels for 1 hour before they were injected with dye, as this procedure had been found to relax the greater vessels (21). These

hearts showed no better injections of the capillaries than did the others injected by the same method, in the absence of potassium sulfocyanate.

Viessens, in his studies of the ducts opening into the heart chambers, made his injections into the coronary arteries, while Thebesius introduced his blowpipe into the coronary vein and observed the bubbles escaping into the immersed chambers. In this study the experiments of both Viessens and Thebesius have been confirmed. Moreover, when perfusion was carried out through the coronary sinus at pressures ranging from 50–150 mm. Hg, almost all of the perfusate ran out through the Thebesian vessels, and in only a few instances did a few drops escape through the coronary arteries. This result was obtained when saline, acacia, agar and gelatin were used. Again in these hearts the capillaries were not injected, thus showing that the perfusate must have escaped by a more direct and larger communication between the veins and the Thebesian vessels, without having passed through the capillaries.

Perfusion was next carried out through the Thebesian vessels. Canulae were introduced into both coronary arteries, the coronary sinus and the other great veins which did not empty into the coronary sinus. All other outlets to the chambers were then carefully tied off after the chambers had been filled with 2 per cent Berlin blue in salt solution. Compressing the heart caused most of the fluid to run out of the veins, a few drops only escaping through the arteries. Histological sections of these hearts also showed little dye in the capillaries. The veins and a few arteries, however, were filled with dye.

These experiments have shown the existence of connections (1) between the arteries and Thebesian vessels and (2) between the veins and Thebesian vessels. In neither instance is the junction made through the capillaries alone. Before discussing the function of these channels it will be of interest to report the results of further experiments.

It will be noted that in all the experiments just described capillaries were rarely filled with the perfusing dye, and in no instance was more than a small fraction of the total number of capillaries injected. This held true even in the hearts where perfusion pressures were raised sufficiently to rupture the arteries.

It was routine practise in these experiments to wash the hearts free

of blood by perfusing them with saline before adding the dye to the perfusate. This procedure invariably distended the walls and dilated the chambers of the non-elastic dead heart. The mere perfusion distended it gradually and the fluid escaping from the Thebesian vessels stretched the walls as the cavities filled. Heyde (22) had shown that slight dilatation of the heart walls resulted in a slowing of capillary flow. These experiments confirmed that finding and failed to fill the capillaries while the heart walls were stretched.

In view of these findings the dye was introduced directly without previous washing out with salt solution in the manner described in the following experiment.

Protocol.

Heart 44.—This heart was obtained 2 hours post mortem and was still warm. Cannulæ were placed in the coronary arteries as quickly as possible and the heart, while the chambers were still collapsed and there was no stretching whatever, was injected with India ink diluted with an equal part of distilled water, heated to 45° at a pressure of 220 mm. of mercury.

The whole heart and aorta immediately became very black and the injection appeared excellent. Special care was taken to get the injection material in at a high pressure suddenly, to avoid distention of the walls of the heart. This is distinctly in contrast to the earlier human heart injections where the hearts were first perfused with salt solution in order to wash out the blood, and in which procedure the chambers were greatly distended. Later perfusion of hearts so injected with dye or ink invariably injected the Thebesian system and very few capillaries.

In this experiment and in all others carried out in the same manner a large number of the capillaries was injected, sometimes as many as 65 to 85 per cent of the total number. In several instances the injection was practically complete in certain areas of the muscle while in other parts the capillaries were only partially filled. It is of great import that the flow from the Thebesian vessels was very small in the hearts that showed such good capillary injection. In the earlier experiments where the coronary arteries were perfused with salt solution before the dye was introduced, it was noted that the capillaries were free from blood shortly after the perfusion was begun. The color of muscle changed from red to pale pink. Hence, when the dye or ink was injected without previous perfusion, it ran into the capillaries

until the heart became distended and then escaped through the Thebesian vessels into the cavities. This seemed the most plausible explanation of the results obtained. On this basis, therefore, the next step in the investigation was clearly indicated, namely, to study the flow from the Thebesian vessels in a beating heart. Consequently, human hearts obtained within 2 or 3 hours post mortem were perfused with oxygenated Locke-Rosenheim (23) solution. Usually they began to beat within a few minutes after the perfusion was started. The following experiment illustrates the method used.

Heart 52.—July 15, 1926. This heart was obtained 2 hours post mortem from the autopsy of boy, age 15, who died from Hodgkin's disease. Heart normal in size. Both coronary arteries were cannulated and a large cannula was tied into the coronary sinus. During this preparation the heart was kept at 37°C. in salt solution. 2 hours and 30 minutes postmortem perfusion of the coronary arteries was started with oxygenated Locke-Rosenheim solution. The temperature of the perfusion fluid was constant at 37°C. and the perfusion pressure was kept at 50–55 cm. of water. Within 5 minutes the auricles began to show very feeble beats and these were followed immediately by beats of both ventricles. A small dose of adrenalin (0.1 cc. of 1:1000) was injected very slowly into the perfusing fluid, and following this the auricles and ventricles began to beat strongly and normally, so that large spurts of fluid were forced from the aorta and the pulmonary artery. The rhythm became regular and the rate became constant at 62 beats per minute. The following flows were then recorded for 1 minute intervals.

After these flows were recorded a suspension of Berlin blue (2 per cent) in salt solution was injected into the perfusion cannulae in the coronary arteries at sufficient rate to replace the Locke-Rosenheim solution. The pressure remained at 50 cm. water. The heart immediately turned to a deep blue color and glacial acetic acid was run into the coronary arteries. The acid stopped the heart beats instantly.

Similar results were obtained in another experiment, the single difference being that the left ventricle remained in systole and did not beat while the other three chambers were beating regularly. The out-flow was measured during 1 minute before and after the heart began to beat.

Perfusion of the coronary arteries with Locke-Rosenheim solution at 50 cm. water pressure 37°C. 3 hours post mortem. The first flow was recorded before the heart began to beat. In this heart the left ventricle did not beat.

When the figures of Tables III and IV are contrasted with those in Tables I and II, the large outflow from the coronary sinus in Tables III and IV is very impressive, and all the more so when it is realized that the outflow from the other great veins not only was not included under the flow from the coronary sinus but was added to the ventricular outflow. In many of the experiments on dead hearts the outflow from the other great veins of the heart equalled that of the coronary sinus. The second column of figures, therefore, contains

TABLE III.

Heart 52.

	From the coronary sinus	From the other veins, Thebesian vessels and leaks	Heart rate per min.
	cc.	cc.	
1st measurement.....	155	295	62
2nd measurement.....	115	225	62

TABLE IV.

Heart. 41. Male. Age 75. Death from Lobar Pneumonia. Heart Normal for Age.

	From the coronary sinus	From the other veins, Thebesian vessels and leaks	Heart rate per min.
	cc.	cc.	
1st measurement.....	205	360	Not beating
2nd measurement.....	215	330	86
3rd measurement.....	125	195	100

part of the venous outflow as well as the Thebesian flow and that part of the perfusate which escaped by leaking from the cut edges. This last source alone—the leaks—averaged much more in Table II than the flow from the coronary sinus and the veins. Of equal importance, too, in this heart was a fact brought out upon microscopic study of the sections from all parts of the muscle. The capillaries were completely and evenly injected (Fig. 4). Thus, in a beating heart, in the absence of dilatation, the capillaries were filled and the Thebesian flow was greatly diminished. The left ventricle which did not beat, was in

rigor and its capillaries were very imperfectly injected in contrast to those of the other chambers which were beating.

The results of these experiments were confirmed by another which differed only in the fact that flows were recorded before and after the heart began to beat. The protocol follows.

Human Heart 53.—Weight 300 gm. Male. Age 52. Death from pernicious anemia. This heart was obtained about 2½ hours post mortem. Cannulae were introduced into both coronary arteries and into the coronary sinus, the latter for recording outflow. At about 3 hours postmortem perfusion with oxygenated Locke-Rosenheim solution was started through the coronary arteries. The temperature of the perfusion fluid was kept at 37°C. at the point of entrance into the arteries. The perfusion pressure was 55 cm. water. As the perfusion was started the chambers filled gradually and there was a very slight dilatation of the right ventricle, which increased gradually until the heart began to beat. Before any beats occurred the outflow was measured during two periods of 2 minutes each. They were as follows:

	Outflow from coronary sinus	Outflow from other veins, Thebesian vessels and leaks
	cc.	cc.
1st measurement	210	525
2nd measurement	190	510

After perfusing about 5 minutes 0.5 cc. adrenalin (1:10,000) was added very slowly to the perfusing fluid. Following this the right auricle began to twitch and immediately both auricles began to beat regularly. Next the right ventricle began to beat and finally the left ventricle joined in and beat very feebly. After another 5 minutes the rhythm was regular and the rate was constant at 78 per minute. At this point the flow was recorded for two more periods of 1 minute each, the results of which follow:

	Outflow from coronary sinus	Outflow from other veins, Thebesian vessels and leaks	Rate per min.
	cc.	cc.	
1st measurement	260	550	78
2nd measurement.....	268	545	78

Before further flows could be recorded the beat of the left ventricle became very weak and finally lost all force so that it merely twitched. At this point Berlin blue 2 per cent was introduced into the coronary artery cannulae at such rate as

to replace the Locke-Rosenheim solution, and the beat of the heart immediately became weaker. Glacial acetic acid was then added to the perfusate and the heart beat ceased. Sections were cut from various parts of the walls, measured and placed in 10 per cent formalin (aqueous) for fixation.

The results of this experiment add to the value of the figures of the previous one (Heart 52) and in addition show a very slight increase of the venous outflow in the beating heart over the outflow before it began to beat. This heart also showed a good capillary injection except in the left ventricle which had stopped beating before the dye was injected.

The important point of this experiment is the relative change in the amount of outflow from the different sources when contrasted to the perfusion of dead hearts. In the beating heart (Heart 52) it did not

TABLE V.
Heart 52 Beating. Heart 24 Not Beating.

	Outflow from Thebesian vessels*	Outflow from coronary sinus and veins	Leaks
	cc.	cc.	cc.
Heart 52.....	140	180	75
Heart 24	305	28	102

* Average of combined outflow from the two ventricles.

seem wise to spend the time required in introducing cannulae into the pulmonary artery, aorta and great veins emptying directly into the right auricle. Consequently, the outflow from the chambers was mixed with leakage from the cut surfaces, and in addition contained the outflow of all the veins emptying into the right auricle except that from the coronary sinus. In many of the perfusion experiments on dead hearts the outflows from the coronary sinus and from certain other great veins were measured separately and the average outflow from the latter was a third to a half of that from the coronary sinus. If one takes that figure as the flow from the other veins and assumes that the leakage was 75 cc. the following values can be calculated for the flows from the different sources.

When these calculations have been made by subtracting the leaks

and venous flow (other than from the coronary sinus) from collected outflow and adding the venous flow to that of the coronary sinus a figure is found which is quite in contrast to those found on perfusing dead hearts.

The evidence thus far considered has been obtained from hearts after death or from isolated beating hearts. In support of this evidence the results of thirty-five experiments on intact cats are offered. The cats were carefully anesthetized with sodium barbital and when completely under the anesthetic, the chest was opened under artificial respiration in such manner that the heart was exposed. A dye or India ink¹ was injected into the left auricle or left ventricle and the heart, by pumping the dye or India ink into the coronary arteries, injected itself. Immediately after the injection of India ink was started, the arteries first filled and appeared as black stripes on the heart. As the ink reached and filled the capillaries the entire heart became uniformly black but remained so only for a brief second or more when it suddenly began to dilate. During the dilatation, even though the ink injection was continuous, the beats became very feeble and the color changed from deep black to a pale pinkish color, as the feeble beats "milked" or squeezed the dye from the capillaries.

An explanation of the change in the color of the heart as it dilated and began to beat feebly was difficult, but it was furnished by the two experiments which follow.

Cat 2.—Weight of cat 3 kilos. May 14, 1924. Anesthesia was induced by means of sodium barbital (0.6 gm. per kilo intraperitoneally). Blood pressure was then registered by means of a cannula in the carotid artery. The chest was opened under artificial respiration and the pericardium incised in such a manner that the left auricle and ventricle came fully into view. India ink diluted with an equal part of distilled water at a temperature of 37°C. was then injected directly into the left ventricle. As the injection began the aorta was clamped at the level of the right carotid artery. The pressure used in injecting the ink was barely sufficient to cause the ink to enter the ventricle against ventricular pressure. When the heart began to turn black, showing a capillary injection, 5 mg. of histamine was injected along with the ink. The total amount of ink used was 20 cc. At a certain time during the injection the heart turned very black and at this

¹ Berlin blue 2 per cent suspension in normal saline. Either Weber's or Higgins' India ink was used, as a 50 per cent suspension in distilled water. Both dyes and inks were filtered immediately before using.

point the large clamp was placed on the heart in the auriculoventricular groove. The vessels at the base were also clamped, the heart excised and placed in 10 per cent formalin with the clamps in place.

This heart ceased to beat almost immediately after the clamp was placed in the auriculoventricular groove. The injection of the capillaries appeared to be perfect on gross examination.

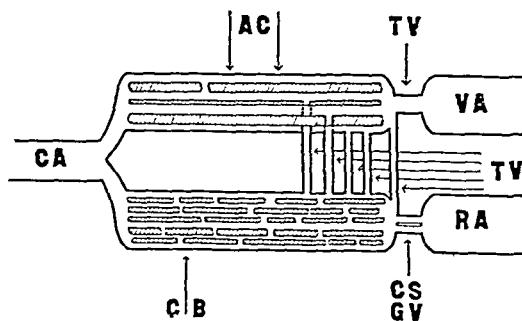
Cat 5.—Weight 3.1 kilos. May 20, 1924. Anesthesia was induced by means of sodium barbital (0.5 gm. per kilo intraperitoneally). Both carotid arteries were cannulated as was also the femoral vein. Blood pressure tracings were made from the left carotid and finally the abdomen was opened and a ligature placed around the aorta but it was not tied. Berlin blue (2 per cent suspension in 0.85 per cent salt solution) was slowly injected into the femoral vein. After approximately 10 cc. had been injected the blood pressure began to fall and the injection was made more rapidly from that time on, but the rate was never such as to raise the blood pressure above the original level.

The chest was hurriedly opened and it was found that the heart had stopped beating in systole and was deep blue in color. Additional Berlin blue was then injected through the right carotid artery after the ligature around the aorta had been tied. The injection pressure was 220 mm. of mercury. 10 per cent formalin was finally run in through the same artery at the same pressure. The chest was opened, the vessels of the heart clamped at the base, the heart excised and placed in 10 per cent formalin.

The important point that was common to these two experiments was the fact that the heart ceased to beat in each instance while the capillaries were filled and before it began to dilate. Furthermore, microscopic sections of both hearts proved the capillaries to be perfectly injected. The explanation to be given of this behavior, while not necessarily a final one, seems entirely reasonable. As the injection of ink was begun it entered the capillaries and cutting off the oxygen to the muscle, caused the heart to dilate. As dilatation occurred the capillaries were stretched and thereby further entrance of ink into them was prevented. But the feeble beats of the dilated heart were sufficient to squeeze the ink out of the capillaries. Such circulation as continued was almost certainly through the Thebesian vessels which were found well injected with the ink when the sections were studied with the microscope. Repeated efforts have been made to duplicate these experiments following each step with the greatest care, but all have failed because it has not been possible to stop the hearts at the proper stage during the injection, while the capillaries were filled. Sections in these later instances showed that only the large vessels were filled.

After witnessing these experiments one can hardly refrain from speculation as to the reason why the cardiac capillaries are filled with such difficulty and what part, if any, the Thebesian vessels play as an impediment to their filling. The results just reported furnish data which will serve as a basis for an hypothesis.

The facts that capillaries are perfectly filled only in a beating heart, that in these experiments at least, they fill less completely in a non-distended dead heart, and scarcely at all in a dilated heart, suggest the Thebesian vessels as a cause for the lack of success in the last two instances. The beating heart therefore must at some stage of its cycle close the Thebesian vessels and force the blood through the capillaries. This is not at all unreasonable when one considers the pressure relations in the left ventricle and the aorta during systole and diastole. If one accepts for the moment the presence of the direct communications between the



TEXT-FIG. 1. *CA* = coronary arteries. *AC* = arterial communications with Thebesian vessels. *TV* = Thebesian vessels. *VA* = cavities of ventricles and auricles. *CB* = capillary bed. *CS* = coronary sinus. *GV* = great veins emptying directly into the right auricle. *RA* = right auricle.

coronary arteries and the heart chambers on one hand and between the veins and chambers on the other, a chart can be drawn which illustrates diagrammatically the various vascular channels of the heart. The commonly accepted route of blood flow in the heart is from the aorta into the coronary arteries and thence through the capillaries into the large veins which empty into the right auricle directly or *via* the coronary sinus. In the diagram in Text-fig. 1, this route would be shown by having the blood flow from the coronary arteries (*CA*) through the capillary bed (*CB*), and the coronary sinus and veins (*CS* and *GV*) into the right auricle (*RA*). The experiments just reported have shown a direct communication between the coronary arteries (*CA*) and the heart chambers *via* the channels (*AC*) to the Thebesian vessels (*TV*). And in dilated dead hearts these channels

proved so readily accessible that approximately 60-90 per cent of the fluid perfused into the coronary arteries escaped through them. In beating hearts the amount was considerably less and more perfusate ran through the capillaries (*C B*) into the coronary sinus and veins (*C S* and *G V*). If the pressure that exists in life during the various phases of the cardiac cycle be considered, a hint of the use of the Thebesian vessels may be found. During ventricular systole the pressure is the same, of course, in the left ventricle and at the mouth of the coronary arteries, but the pressure in the right ventricle and in both auricles is less than it is in the coronary arteries. In diastole, however, with the aortic valves closed, the pressure in the coronary arteries (*C A*) is greater than in any of the heart chambers, hence an increased outflow from the Thebesian vessels (*T V*) into the heart chambers (*V A*) would be expected. Even during ventricular systole there is possibly a little outflow into the right ventricle, and an unhindered flow into the auricles. The number of openings in the auricles, however, is usually small.

This set of pressures would certainly retard the flow from the coronary arteries (*C A*) to the right ventricle and stop it from the coronary arteries (*C A*) to the left ventricle; and would, therefore, greatly inhibit the flow from the coronary arteries (*C A*) to the chambers (*V A*) during systole. The alternate route of flow would be through the capillary bed (*C B*). The Thebesian system, therefore, would serve as an additional means of escape of the blood in the heart wall and thereby enable the coronary vessels to empty themselves very rapidly. The veins also communicate directly with the Thebesian vessels, therefore the latter might serve as a means of rapid emptying of the entire vascular bed of the heart during diastole. Moreover, since some of the Thebesian vessels are connected directly with the capillaries, it may be possible for blood to flow from the ventricle through these vessels into the capillaries.

Such speculations as have been discussed are interesting but extremely difficult of proof. They are offered as possible explanations with full realization that further work is indicated which may or may not support them. So long as they are recognized as theory rather than fact they may serve to stimulate further study of the function of these vessels in normal hearts.

Finally another very important function of the Thebesian vessels has been demonstrated by the findings in two hearts at the post-mortem table.² This function has been proved more conclusively by the clinical histories and necropsy findings in these two cases than it could be by any experiment in the laboratory. In each of the hearts

² It was possible to study and use these hearts through the kindness of Dr. Timothy Leary. The cases are being reported in detail in another paper.

there was complete closure of both orifices of the coronary arteries. The first one was found in a negro woman, about 26 years of age, who had worked and earned her living as a seamstress up to within a few days of her death. She was found dead in her room and as she lived alone the nature of her death could not be learned. A syphilitic process in the aorta, probably of long standing, had completely closed and obliterated the orifices of both coronary arteries.

The second heart was from a man 35 years old who at the time of his death was working as a seaman. It was learned that he had been considered very lazy by other members of the crew but he was able to do his work and so far as they knew, did it without any symptoms or signs of heart disease. Between his jobs as a seaman he worked for an awning company and assisted in putting up and removing awnings from houses. In this heart also there was a definite syphilitic aortitis which had closed the orifices of both coronary arteries.

Needless to say, in both instances a very careful search for other openings of the arteries was made, but without success. Attempts to inject fluid and pass probes through the orifices also failed. Moreover, sections through the vessels $\frac{1}{2}$ cm. outside the aorta revealed normal lumens. The orifices had, therefore, been closed by the disease in the aorta, and the closure almost certainly had been a very gradual one. Sections through the arteries at their orifices showed complete closure in all vessels and in these the process was of long standing.

In each case a heart without openings for the coronary arteries had maintained an efficient circulation which enabled the person to earn a living. The obvious questions are where and how did these hearts get sufficient circulation to function so efficiently? The answer is not difficult for the only other entrance to the coronary circulation is through the Thebesian vessels and in the instances just cited, at least, they were able to supply the heart muscle with the necessary blood to maintain a wage-earning life. It should be emphasized at this point that the closure of the coronary orifices in these two cases was a gradual one. It was this time element, most probably, that enabled the Thebesian circulation to take over the new duties and perform them so efficiently. One does not need to search far afield for evidence to support this claim. It is commonplace to find at necropsies extensive

sclerosis of the coronary arteries, so well advanced that it is difficult to introduce the point of a pin into the calcified lumens. In many instances these people had been comparatively well and healthy individuals, with dyspnea on exertion perhaps, but falling into the group of men considered normal at their age. In such conditions it is certain that the process of occlusion was a gradual one. Their clinical histories stand out in sharp contrast to those of patients with sudden closure of one branch of a coronary artery. Many of these die at the time of the closure while others recover after acute heart failure. In certain emergencies, therefore, particularly when allowed sufficient time to adapt themselves, the Thebesian vessels can take over the function of the coronary arteries.

SUMMARY.

In summary, evidence has been presented to show a direct connection other than through the capillaries between the coronary arteries and the chambers of the heart.

This connection was shown by perfusion, injections and serial sections to be through the Thebesian veins. Communications between the larger coronary veins and the Thebesian veins were also demonstrated by the same methods.

Serial sections through Thebesian veins have shown capillaries draining directly into them. Under certain conditions it has been shown that as much as 90 per cent of the arterial flow may escape *via* the Thebesian vessels.

Lastly, in the event of gradual closure of the orifices of the coronary arteries, the Thebesian vessels can supply the heart muscle with sufficient blood to enable it to maintain an efficient circulation.

This work has been in progress for 4 years during which time Dr. William B. Stevens assisted in many of the earlier experiments. I wish to express my deep appreciation to Misses Olivia Ames and Sylvia Warren, and Drs. Henry Jackson, Jr., and Joseph M. Hayman, Jr., for their assistance in translating the older French and Latin papers; and to Dr. Francis W. Peabody for his very helpful criticisms and advice throughout the investigation.

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EXPLANATION OF PLATES.

PLATE 17.

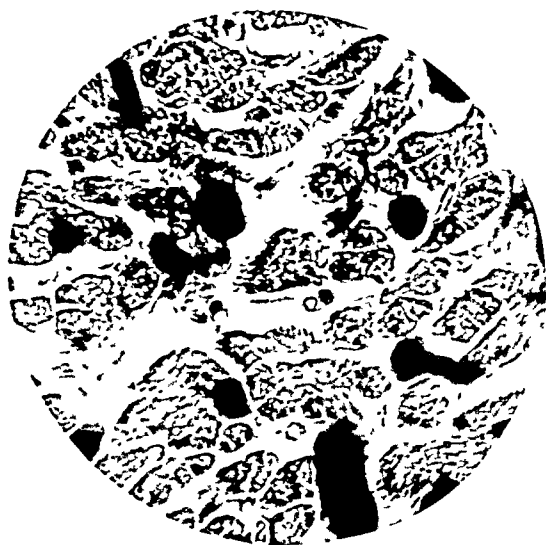
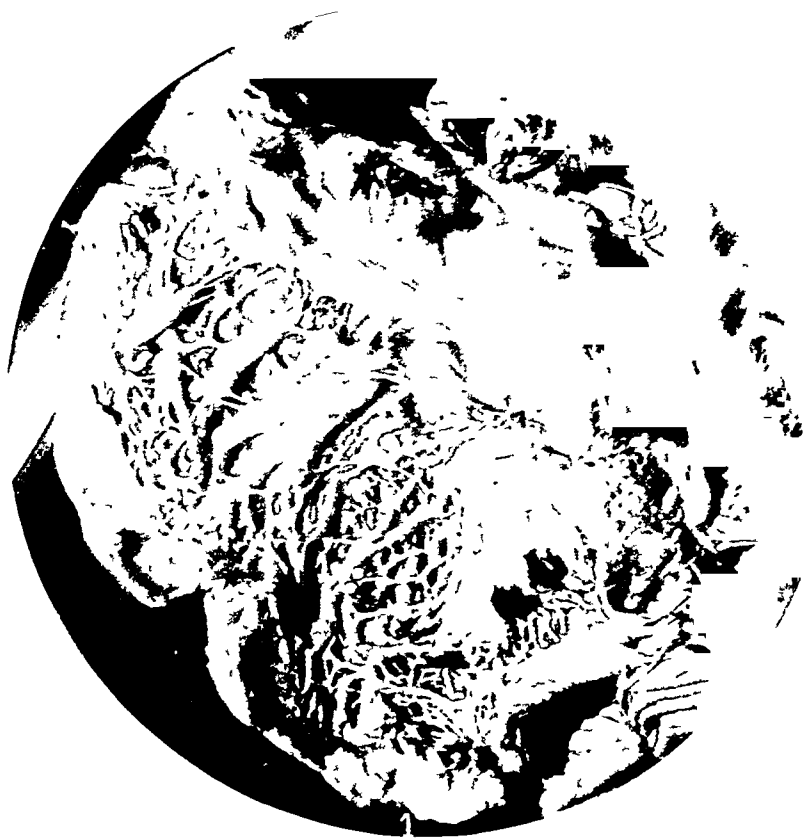
FIG. 1. Left ventricle of heart injected with celloidin, showing the celloidin plugs protruding from the Thebesian vessels. The plugs have been covered with white ink in order to make them show more distinctly in the photograph.

FIG. 2. Cross-section of muscle fibres from the left ventricle of Human Heart 14 showing the larger vessels well filled, but scarcely any capillary injection.

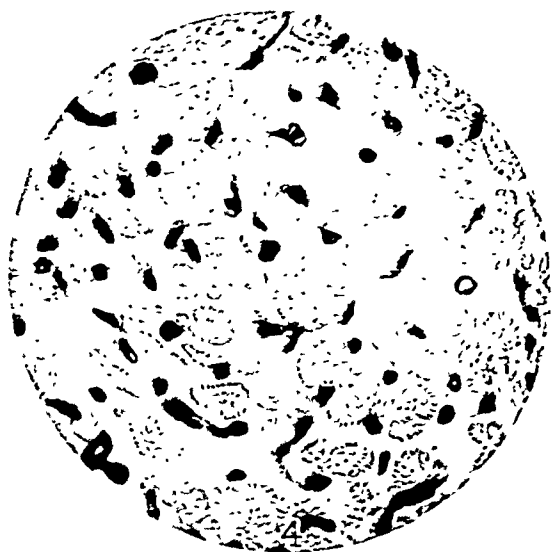
PLATE 18.

FIG. 3. One of a series of sections through a Thebesian vein (the channel running across the middle of the field) showing a vein and capillaries emptying into the Thebesian vein. This section lay less than 0.5 mm. below the endocardial surface.

FIG. 4. Cross-section of muscle fibres and capillaries of right ventricle of Human Heart 41 injected while beating.



(Worm Thelohan vessels in heart circulation)



(Wearn: Thebesian vessels in heart circulation.)

A STUDY OF THE MECHANISM OF RECOVERY FROM EXPERIMENTAL PNEUMOCOCCUS INFECTION.

BY OSWALD H. ROBERTSON, M.D., SHUTAI T. WOO, M.D., SHEO
NAN CHEER, M.D., AND LI PIN KING, M.D.

(From the Department of Medicine, Peking Union Medical College, Peking, China.)

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In previous investigations of the nature of natural immunity to pneumococcus infection (1-3) evidence of a defense process common to both pneumococcus-resistant and susceptible animals was found. It was possible to demonstrate, by appropriate methods, pneumococidal activity in varying degrees with serum-leucocyte mixtures of the different animal species studied. The relatively marked pneumococcus-killing power of the naturally immune animals' blood was found to depend on the presence of opsonins capable of causing the phagocytosis and destruction of highly virulent pneumococci. The blood of susceptible animals lacked this property of influencing virulent strains but exhibited opsonic activity and destroying power for pneumococci of low virulence for the species. In relation to the total resisting capacity of the body, the degree of potential pneumococcus-killing energy resident in the blood suggests that it is an important if not the principal means of normal antipneumococcus defense. The question then arises as to whether recovery from pneumococcus infection is brought about by a similar mechanism or whether other and quite different forces are employed by the body in terminating the activity of pneumococci which have succeeded in overcoming the initial resistance of the animal. The results of a study of this problem by the same methods employed in the previous work constitute the subject of the present communication.

A review of the recent literature on immunity factors in recovery from experimental pneumococcus infection reveals several points of view which have arisen as a result of a diversity of experimental findings. Certain investigators, Bull (4), Tchistovitch (5), Tudoranu (6), and others, believe that recovery is

brought about by the elaboration of specific immune substances, agglutinins, opsonins, or protective bodies which promote the destruction of the disease producing microorganisms by the leucocytes. Other workers take an entirely opposite view, namely that recovery is quite independent of these manifestations of humoral immunity. Singer and Adler (7) eliminate humoral immunity completely from the mechanism and hold that a specific immune change occurs in the cells of the reticulo-endothelial system by which they are enabled to engulf and destroy the invading pneumococci. Wadsworth (8) considers that the production of humoral immune substances is an important element in the recovery process but is of the opinion that pneumococcus immunity is chiefly antitoxic in nature. The majority of investigators, however, have come to the conclusion that the elaboration of antibodies of the bacteriotropic or protective type constitutes a part of the process of acquired resistance but that there are other undetermined factors which play a contributory, and at times a preponderating rôle.

It has been found repeatedly that active immunity may occur in the absence of demonstrable serum immune properties. Cecil and Blake (9) failed to find any constancy in the appearance of protective substances in the blood of monkeys recovering from pneumococcus pneumonia or any correlation between the degree of active immunity of the animal and the passive immune properties of its serum. Wright (10), studying experimental pneumococcus infection in rabbits, observed that the development of opsonic and protective properties was irregular and uncertain and did not appear to parallel the acquisition of active immunity. Other authors have made analogous observations.

This inconstancy in the finding of immune bodies in the serum of animals with acquired resistance appears to depend to some extent on the type of invading pneumococcus. While Cecil and Blake found protective substances occasionally after infection with Types I and II, none were observed to occur in the serum after pneumonia due to *Pneumococcus* Types III and IV. Differences in antibody response to the several pneumococcus types are brought out more clearly by active immunization. In contrast to the comparative ease and regularity with which it is possible to produce immune substances against Types I and II in the different animal species is the uncertainty of antibody response following immunization with Type III. In a recent study Tillett (11) found that only four of twenty-eight rabbits immunized with Type III pneumococcus showed type-specific antibodies in the serum. Tudoranu, however, considers that the difference between immune sera Types I and II and serum Type III is one of antibody concentration only and if enough serum of a Type III immunized or recovered animal is used, specific protection can be demonstrated against the homologous pneumococcus. Felton (12) has thrown further light on this subject by finding that in antipneumococcus serum or antibody solution there is an inhibitory or antagonistic substance which can be eliminated by fractional precipitation. He was able to show that a Type III antiserum thus treated had a much augmented protective power.

The appearance of serum immune bodies appears to be conditioned even more by the kind of animal employed in the study, than by the type of pneumococcus used. Those authors who have found opsonic activity constantly in the serum of animals recovering from pneumococcus infection (Bull and Tchistovitch) worked with a highly resistant species, the dog. Wadsworth, studying the problem, failed to find increased phagocytic power in dogs' blood regularly, but he never found any evidence of this reaction in infected rabbits. The majority of those workers whose findings have lead them to question or reject the probable causal relationship between the occurrence of humoral immunity and recovery have worked with animals highly susceptible to pneumococcus, chiefly rabbits and monkeys.

Among the experimental data of the above authors on pneumococcus infection in susceptible animals one finds occasionally suggestions that there may be some relationship between the degree of active immunity and the presence of demonstrable immune properties. Wright, for example, found evidences of humoral immunity, especially the pneumococidal power of the blood, most constant and most marked at a time when the resistance of the animal (rabbit) was greatest. More recently Stillman (13) made the observation that rabbits exposed repeatedly to sprayings with *Pneumococcus* Type 1 developed protective properties in their serum, the percentage incidence of which increased progressively with the number of sprayings. Since the per cent mortality among the rabbits diminished with the number of exposures to pneumococci it is highly probable that the surviving animals had developed an increased resistance.

In taking up the present work, namely an attempt to determine the part played by humoral immunity in the mechanism of recovery from experimental pneumococcus infection, it seemed wisest to investigate first the reaction of resistant animals since they have been found to yield more constant demonstrable evidence of antibody production; then in the light of the findings which might result from such a study to proceed to observations on a susceptible species.

Methods.

Disease Production.—Cats and rabbits were infected, for the most part by the intrapleural injection of actively growing and abundant broth cultures of pneumococci. A few animals were injected intraperitoneally. The organism used principally was a Type I pneumococcus originally isolated from the blood of a case of lobar pneumonia. After passage through twelve cats its virulence for these animals was found to lie between 0.005 cc. and 0.1 cc. broth culture. Rabbits were killed in the amounts of 0.000,000,1 cc. and mice with 0.000,000,01 cc. Passage through a cat was repeated every month or two during the course of the work. A Type II and a somewhat less virulent Type I pneumococcus were

also used in rabbits. The former killed in doses of 0.1 cc. to 0.01 cc., the latter with 0.000,01 cc. to 0.000,001 cc. broth culture.

Disease Course.—The cats which recovered ran a febrile course usually from 4 to 5 days terminating often with an abrupt fall in temperature. Occasionally the fever persisted for a longer period. A well marked loss in weight was a constant accompaniment. This usually ceased with the fall in temperature but in some animals there was a slight further weight loss followed by a slow subsequent gain, in spite of their appearing otherwise well. The course of infection in rabbits was much more irregular and fluctuations in temperature were found to be less significant than loss in weight. The experimental animals were observed from weeks to months.

Pathology.—The lesions produced in cats by intrapleural inoculation were found to vary widely. Autopsy after fatal termination of the infection showed, in the majority of instances, empyema of one or both sides often with pericarditis and sometimes peritonitis. In only one case was lobar pneumonia observed. Several animals showed no gross pathology, but blood cultures were positive. Some of the cats were killed during and immediately after recovery. Except in one instance in which organized fibrin was observed over the pericardium, no gross lesions were found in these animals. Cats dying after intraperitoneal injection showed peritonitis but no pleuritis. In the rabbits which died, a diffuse peritonitis and pleuritis were found. Lobar pneumonia was not seen. Microscopical studies of the tissues were not made except to verify the presence of pneumonia. Secondary infection with streptococci or Gram-negative bacilli supervening either during the course of infection or after recovery was not uncommon.

Serum.—The test serum was allowed to separate in the cold, then cleared by centrifugation and immediately inactivated at 56° for $\frac{1}{2}$ hour. All specimens from a single animal, kept on ice in sealed tubes, were tested at one time. The dilutions indicated in the tables represent final dilution in the serum-leucocyte mixture.

Pneumococcidal Tests.—The serum of the infected animals was tested for its power to promote pneumococcus killing by adding it to normal rabbit serum-leucocyte mixtures seeded with virulent pneumococci. The technique was the same as that described in a previous paper in the study of this property of specific antipneumococcus serum (14). The infected animals' leucocytes were not used because it was not possible to obtain sufficient cells for the tests without withdrawing injuriously large quantities of blood and in the experiments with cats, normal leucocytes could not be employed since it would have been necessary to add normal active cat serum which mixture possesses pneumococcus-killing power. Heating the serum annuls this action. By using the serum-leucocyte mixture of a normal susceptible animal which of itself has no pneumococcidal action, any degree of this property occurring can be attributed directly to the effect of the added infected animal's serum. The amount of pneumococcus suspension

with which the serum-leucocytes were seeded was chosen arbitrarily as containing a sufficient number of pneumococci to produce abundant macroscopic growth in 15 to 18 hours but still few enough to permit effective action of the immune serum in high dilution.¹

Opsonic and Agglutination Tests.—The serum was tested for opsonins and agglutinins according to the technique described in a preceding paper (3). The ratio of serum to pneumococcus suspension employed was 20:1.

EXPERIMENTAL.

Cats.

Development of Pneumococcal Promoting Properties in the Serum Following Infection and Their Relation to Active Immunity.

It was found constantly that the serum of cats recovering from experimentally induced pneumococcus infection possessed the power to promote destruction of pneumococci in rabbit serum-leucocyte mixtures analagous to that caused by the presence of specific anti-pneumococcus serum. This property or substance was heat-stable and could be demonstrated in high dilutions of the serum. The method of testing such sera is shown in Table I in which are given the details of the tests made on the serum specimens of Cat 1, Text-fig. 1, secured before and after infection. It is seen that the presence of inactivated normal cats' serum had no detectable retarding effect on the growth of pneumococci in rabbits' serum and leucocytes even in dilution of 1:10 (lower dilutions could not be used on account of the disturbing effect of cytotoxic action), whereas the serum of the same cat obtained after recovery from a well marked infection, conferred pneumococcus-killing power on these mixtures in dilution as high as 1:5120. The immune horse serum tested at the same time as a control on the activity of the rabbit serum and leucocytes showed a potency ten times as great.

Following recovery the cats were found to be resistant to many times the fatal dose of pneumococci.

¹ In a previous paper (14) it was found that there exists a quantitative relationship between the concentration of immune serum present and the number of pneumococci destroyed by the serum and leucocytes.

Appearance of Serum Immune Properties in Relation to Temperature and Blood Invasion.

Tests on samples of blood taken frequently during the course of infection showed that in those animals destined to recover, serum immune properties appeared about the 4th day of the disease (Text-figs.

TABLE I.

Determination of Pneumococcal Promoting Power of Cat's Serum before and after Pneumococcus Infection.

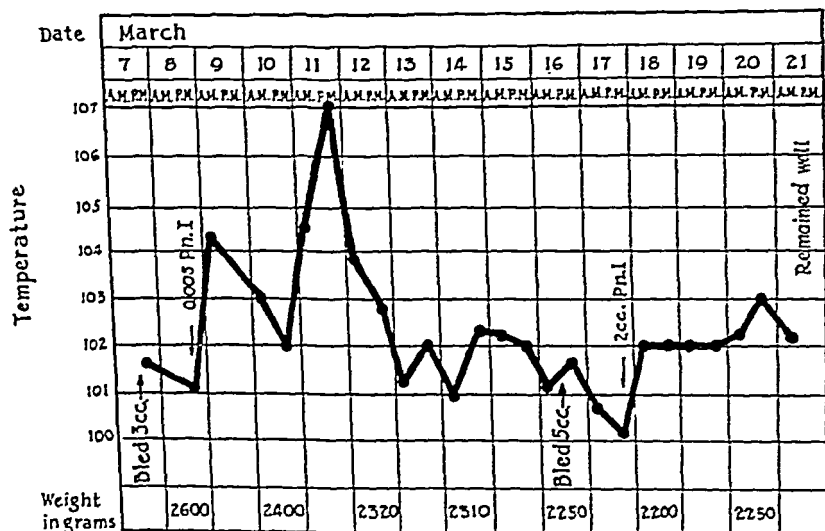
Normal rabbit serum 0.2 cc. + rabbit leucocyte suspension 0.1 cc. + diluted cat serum 0.1 cc. + pneumococcus suspension 0.1 cc.

	Amount of standard pneumococcus suspension	Dilutions of cat serum	Growth as shown by color change at hrs.*				Pneumococci in stained film at 72 hrs.
			15	18	24	48	
	cc.						
Cat's serum before inoculation	0.000001	1:10	++++				+
	"	1:20	++++				+
	"	1:40	++++				+
	"	1:80	++++				+
Cat's serum after recovery	"	1:320	0	0	0	0	0
	"	1:640	0	0	0	0	0
	"	1:1280	0	0	0	0	0
	"	1:2560	0	0	0	0	0
	"	1:5120	0	0	0	0	0
	"	1:10240	0	0	++		+
		Dilutions of immune horse serum					
Controls with immune horse serum	"	1:6400	0	0	0	0	0
	"	1:12800	0	0	0	0	0
	"	1:25600	0	0	0	0	0
	"	1:51200	0	0	0	0	0
	"	1:102400	0	0	+++		+
Controls with normal rabbit serum and leucocytes only	"		++	++++			+
	"		+++	+++++			+
	0.0000001		+	++++			+
	"		++++	+++++			+
	"		++++	+++++			+

* Degree of methemoglobin formation.

2, 5, and 6). In two cases they were demonstrable on the 3rd day. The titer of the pneumococcidal promoting substances rose abruptly and usually reached its highest point within 48 hours where it tended to remain comparatively stationary for several days before beginning a gradual drop. Coincident with the appearance of immune bodies in the serum there occurred in most instances a sharp drop in temperature, although often it did not reach normal until several days later.

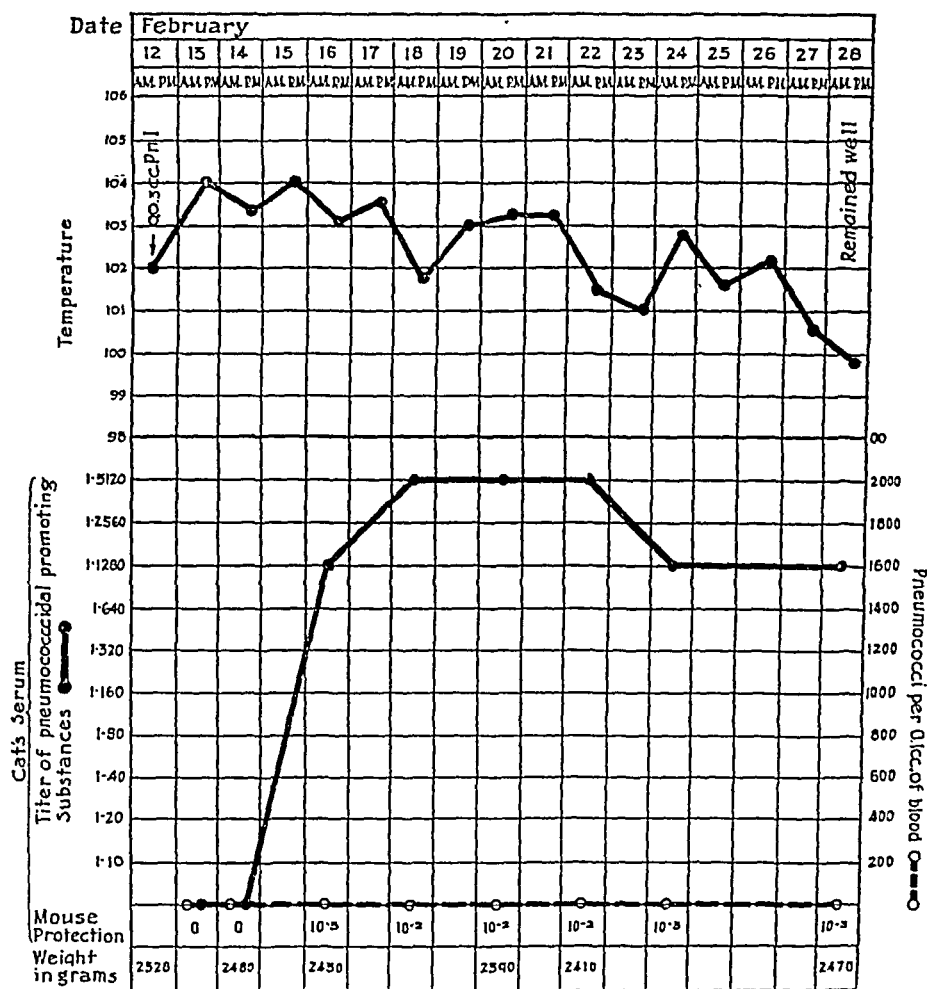
Blood invasion which was present in the majority of the infected cats and often to an intense degree, terminated abruptly with the



TEXT-FIG. 1. Cat 1. Experimental pneumococcus infection following the intrapleural injection of 0.005 cc. of *Pneumococcus* Type I broth culture.

appearance of pneumococcidal promoting substances in the blood (Text-figs. 3, 5, and 6). In most instances this immune property was not demonstrated until the blood was sterile, but occasionally as shown by Cat 5, Text-fig. 5, both antipneumococcus substances and pneumococci were found to be free in the blood stream at the same time. It is possible that had blood specimens been taken with sufficient frequency this transient simultaneous presence of circulating

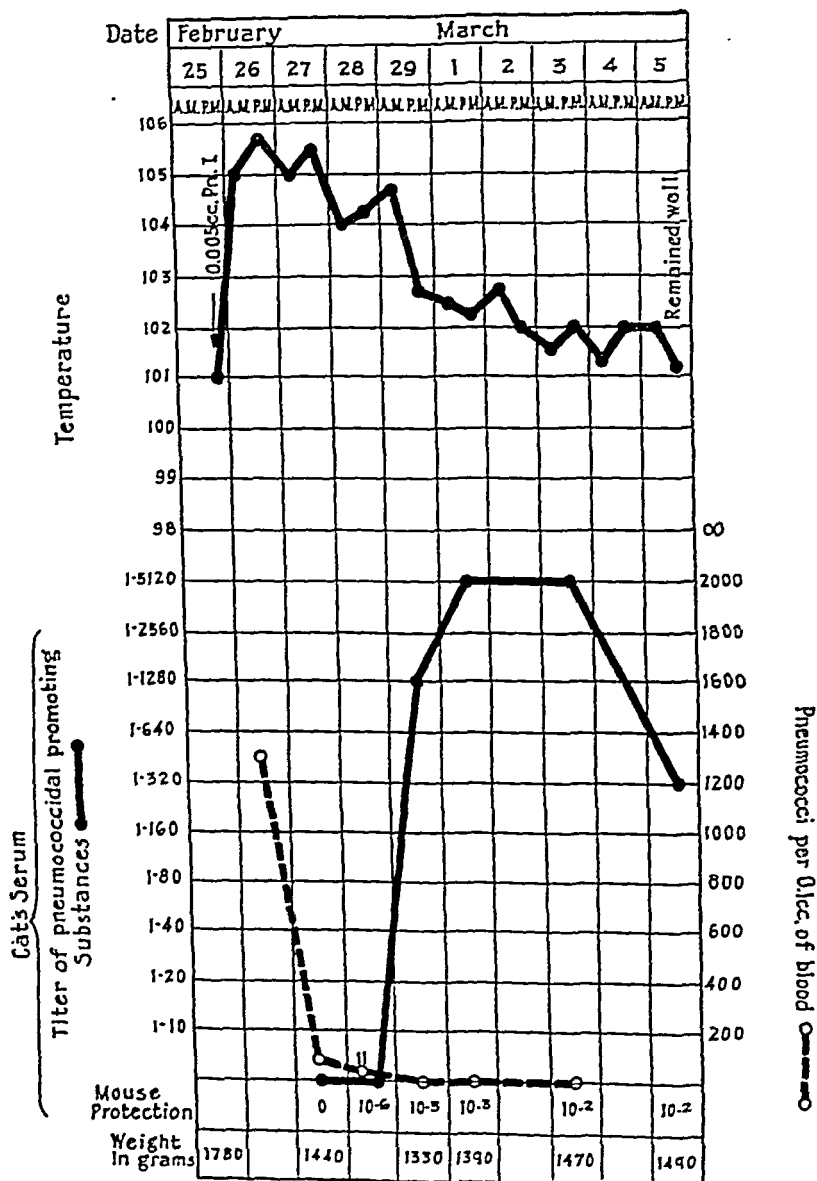
antigen and antibody would have been observed much more frequently or perhaps regularly. The constant and marked decrease in the number of colonies shown by blood plates during the 24 to 48 hours



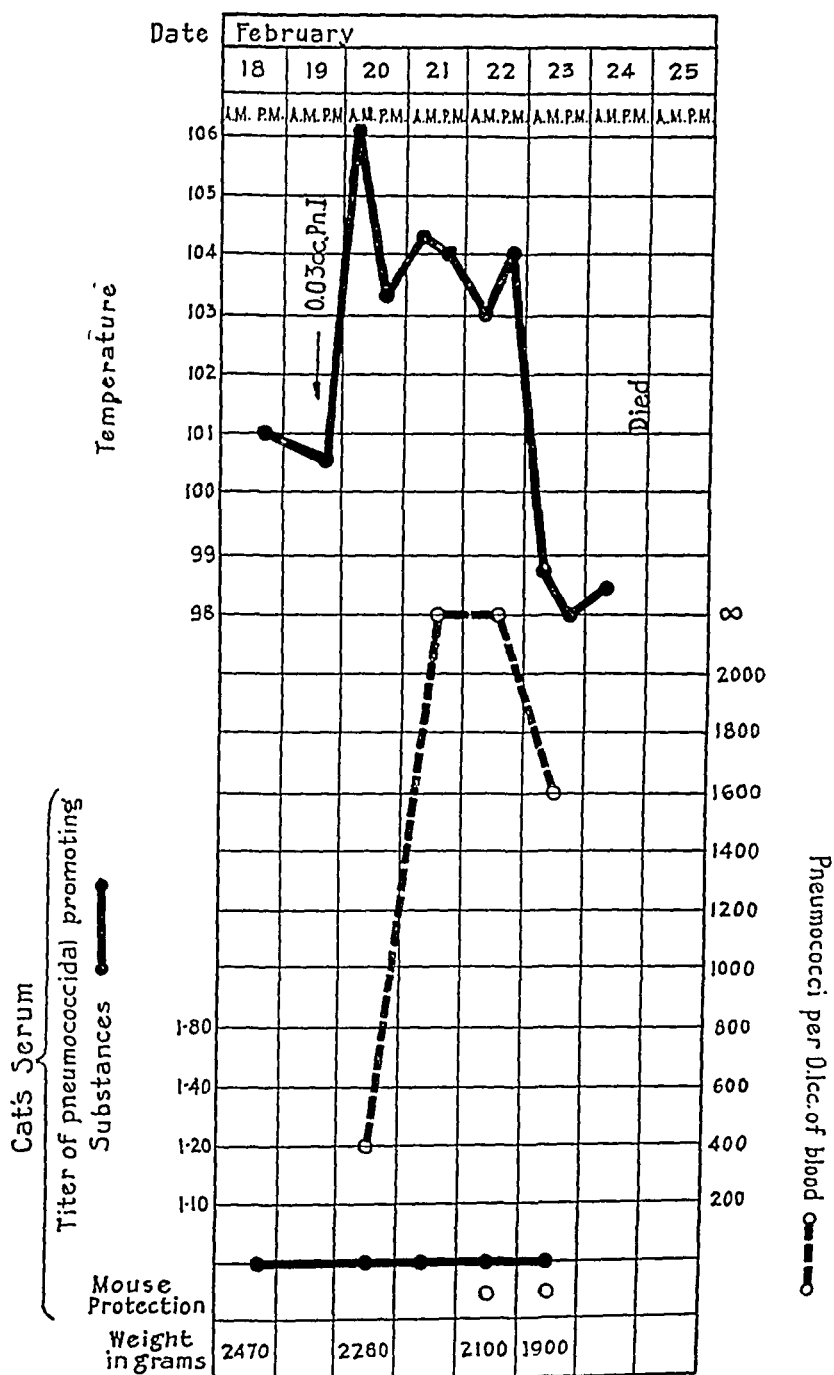
TEXT-FIG. 2. Cat 2. Experimental pneumococcus infection following the intrapleural injection of 0.03 cc. broth culture, *Pneumococcus* Type I.

preceding the appearance of immune substances in the blood, suggests that the elaboration of these bodies begins early in the disease.

All those cats in which the disease went on to a fatal termination

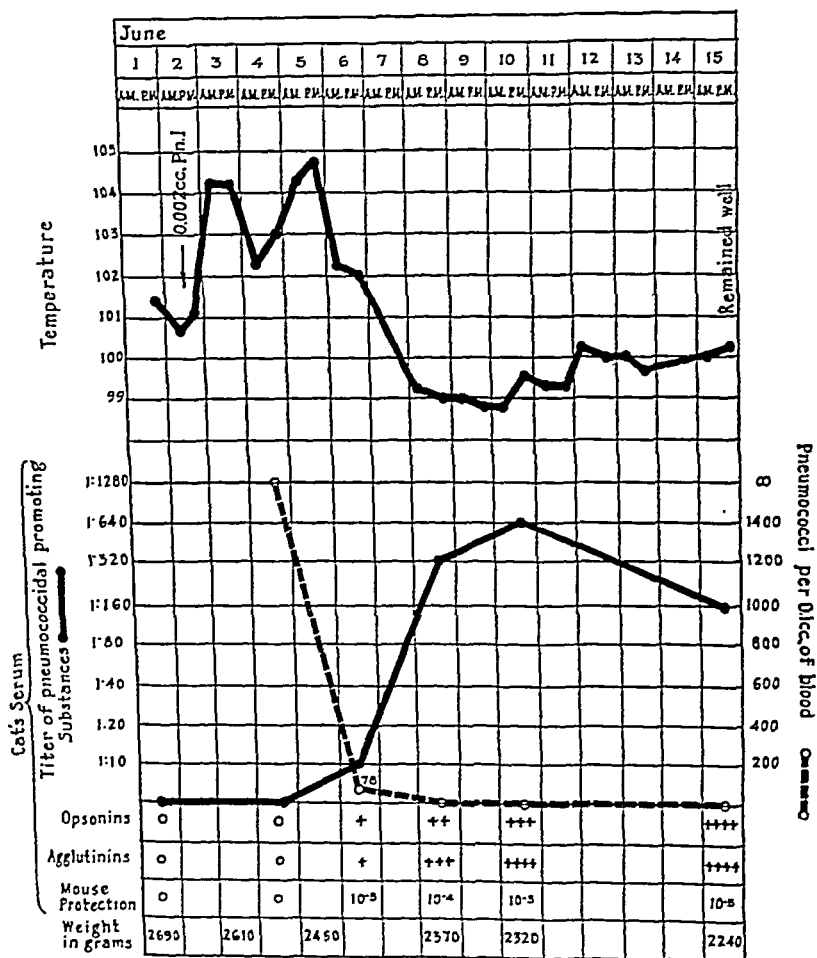


TEXT-FIG. 3. Cat 3. Experimental pneumococcus infection following the intrapleural injection of 0.005 cc. broth culture, *Pneumococcus* Type I.



TEXT-FIG. 4. Cat 4. Experimental pneumococcus infection following the intrapleural injection of 0.03 cc. broth culture, *Pneumococcus* Type I.

failed to develop demonstrable serum immune properties and showed a persistent marked bacteriemia (Text-figs. 4 and 7).



TEXT-FIG. 5. Cat 5. Experimental pneumococcus infection following the intraperitoneal injection of 0.002 cc. broth culture, *Pneumococcus* Type I.

Character of Immune Bodies Occurring at Recovery.

In a number of instances serum specimens were also tested for their opsonic, agglutinative, and mouse protective actions. The results of

one such complete study are shown in Text-fig. 5. A very close parallelism was found to exist between the appearance of these three well recognized evidences of antipneumococcus reaction and the pneumococcal promoting power of the serum. With very few exceptions they all appeared at the same time and increased together although not necessarily in a strictly quantitative relationship. In two cases mouse protective properties² were demonstrable before pneumococcal promoting power became apparent. Text-fig. 3 shows one such instance. A repetition of the test gave the same result. And in one other cat the serum showed slight opsonic activity the day preceding the detection of the other evidences of immune change. The absence of exact parallelism between the appearance and rates of increase in intensity of these several reactions affords, however, no objection to the supposition that they represent different manifestations of one immune response since the various methods employed to bring them out are not comparable quantitatively. During the initial period of recovery when the concentration of immune substances was low, frequently neither agglutinative nor opsonic activity could be demonstrated with equal parts of serum and pneumococcus suspension but could be brought out clearly when a ratio of twenty parts serum to one part pneumococcus suspension was used.

The animals which died failed to show at any time during the course of the disease any of the above studied serum immune properties.

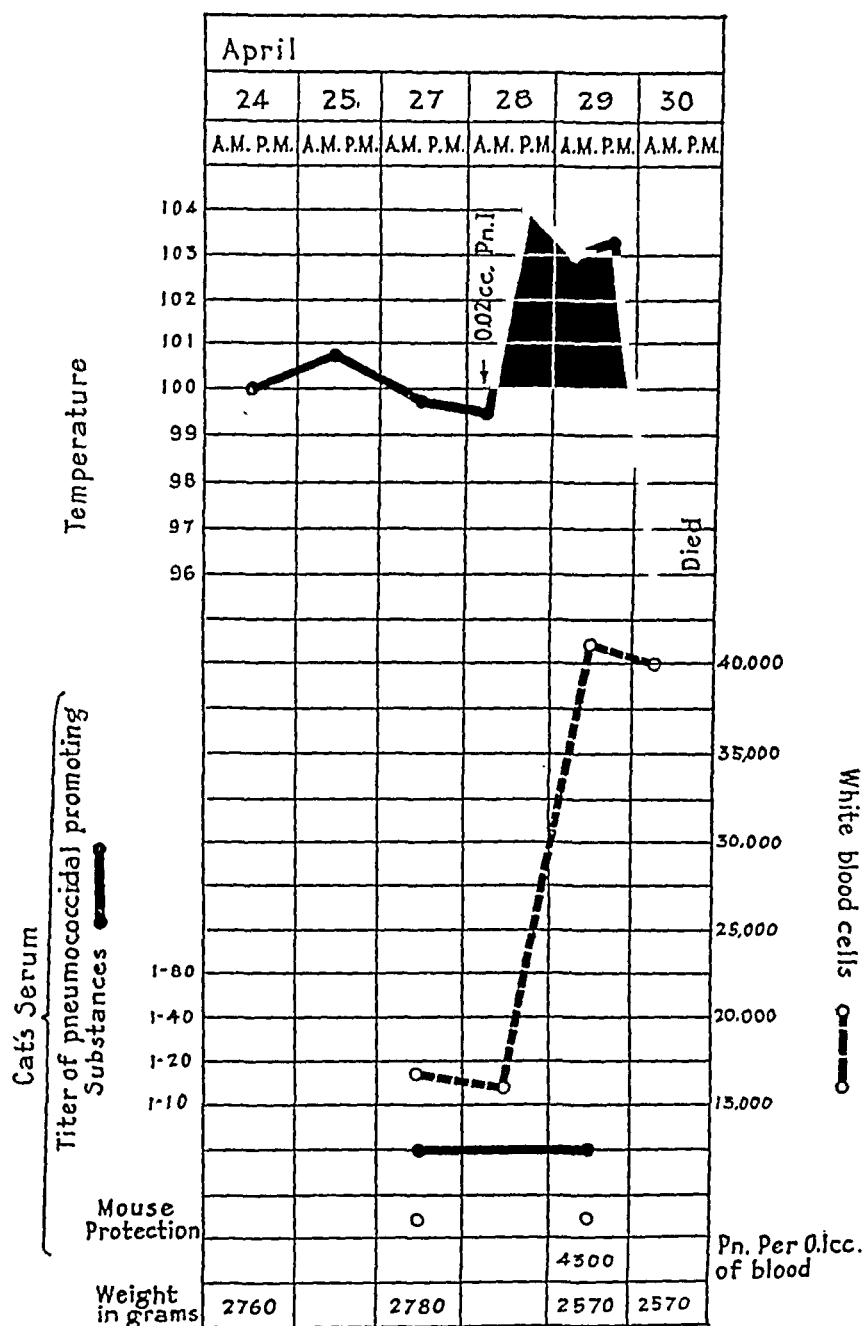
Specificity of Serum Immunity.

Tests carried out to determine the specificity of the immune response in the serum of recovered cats showed that such serum was strictly type-specific as to pneumococcal promoting, agglutinative, and opsonic properties.

Relation of Leucocyte Count to Course of Disease.

Estimation of the number of circulating white blood cells made at frequent intervals during the experimental disease, failed to reveal any constant difference in leucocyte response between the animals recovering from infection and those which succumbed. After an

² The figures recorded on the charts indicate the dilution of culture against which 0.2 cc. of the animal's serum protected.



TEXT-FIG. 7. Cat 7. Experimental pneumococcus infection following the intraperitoneal injection of 0.02 cc. broth culture, *Pneumococcus* Type I.

infection (see chart of Cat 7, Text-fig. 7). Another cat (chart not exhibited) showed a count rising from 33,000 on the 1st day to 50,000 on the 4th day when death occurred. Still another died on the 8th day with the white blood cells at 42,000. Counts as high as 85,000 were observed within 2 or 3 days of death. In certain of these animals showing a terminal high white count empyema was found, but in others there was no evidence of complications.³

It is evident from these observations that the course of pneumococcus infection in the cat is not determined primarily by the number of circulating leucocytes. In the absence of detectable serum immunity the leucocytes, no matter in how great numbers present, seem to have no inhibiting effect on either blood invasion or progression of the disease. Cat 6, Text-fig. 6, showed a rapidly increasing number of pneumococci in the blood in spite of a leucocyte count of 60,000, and it was not until humoral immune bodies appeared that blood invasion ceased. It was noted repeatedly that with immune substances present in the blood a moderate increase only in the number of leucocytes appeared to be compatible with rapid and complete recovery.

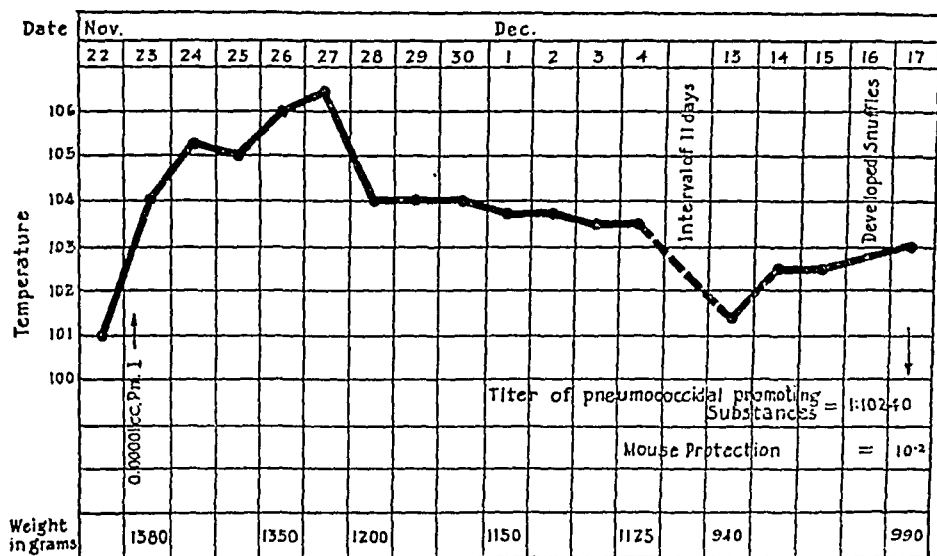
While the scope of this work did not include a complete study of the duration of active and passive immunity following infection, observations were made on three cats after a lapse of 2 months. Just prior to reinoculation with 10 to 20 times the killing dose of pneumococci two of the cats showed a low titer of pneumococcidal promoting substances in the serum, the third showed none. All three animals reacted with a marked rise in temperature and weight loss but recovered in 3 to 4 days. In striking contrast to the behavior of these animals is the absence of any appreciable reaction observed in cats reinoculated soon after recovery from infection when the concentration of serum immune substances was at a high level (see charts, Text-figs. 1 and 6). These were two of the cats used for the tests 2 months later.

Rabbits.

Attempts to study in rabbits the type of experimental pneumococcus infection observed in cats were found to be attended by many

³ Furthermore the development of empyema was not always accompanied by a rise in the number of leucocytes.

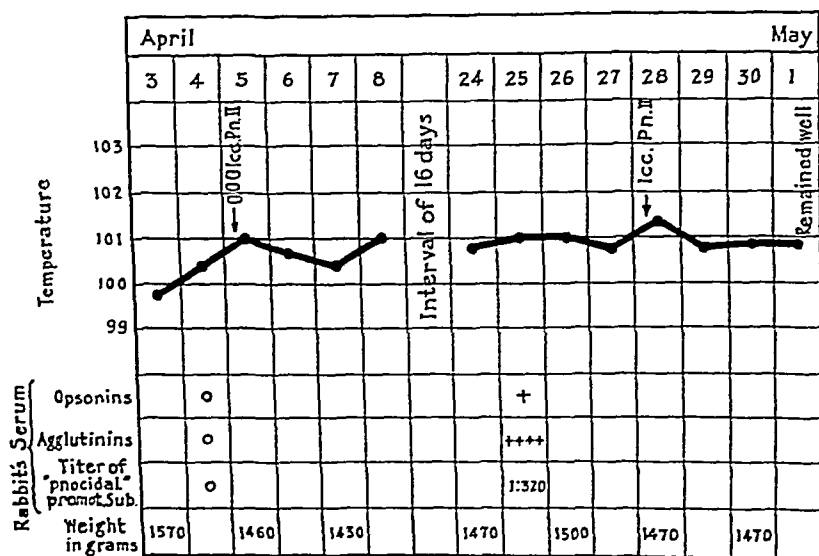
difficulties on account of the wide individual variations in susceptibility which rabbits manifest towards the pneumococcus. A great majority of the animals inoculated, either died or failed to show evidence of disease. Furthermore, withdrawal of blood in any quantity during the stage of active infection had very disturbing and often disastrous effects. From a very large number of trials a few satisfactory observations were secured but these were by no means as complete as those made on cats.



TEXT-FIG. 8. Rabbit 1. Experimental pneumococcus infection following the intrapleural injection of 0.000,01 cc. broth culture, *Pneumococcus* Type I.

The main findings in cats as regards the occurrence of immune substances in the blood, were confirmed in the rabbit. Following recovery from experimental pneumococcus infection, there appeared in the rabbit's serum pneumococcal promoting substances which were shown to be associated with the acquisition of greatly increased pneumococcus resistance (Text-figs. 8, 9, and 10). Likewise opsonic, agglutinative, and mouse protective properties were demonstrable in the serum at the same time. The intensity of the immune response appeared to be fully as great in rabbits as in cats when similar experimental conditions obtained.

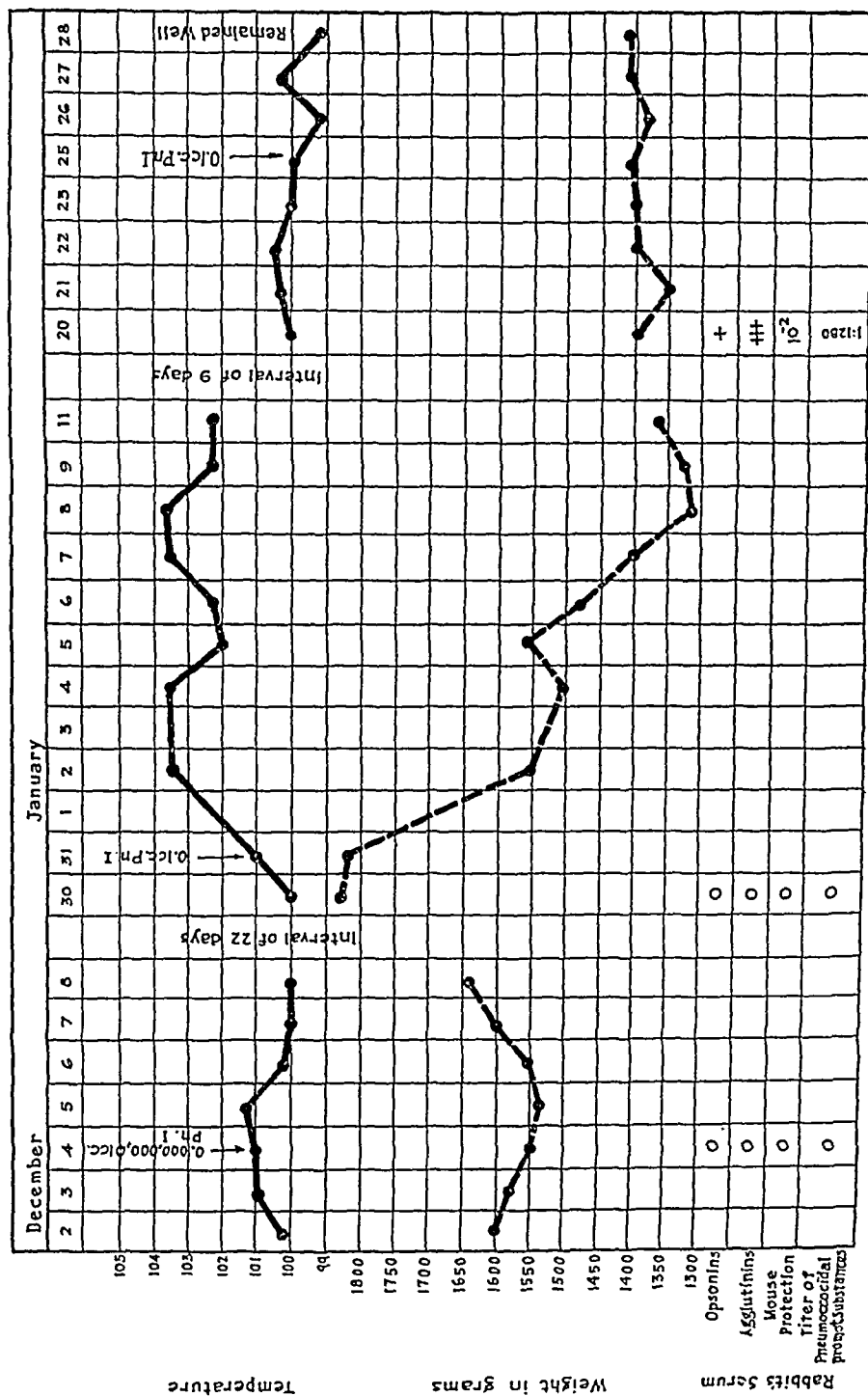
The charts of the three rabbits exhibited exemplify several types of reaction to pneumococcus infection which differ considerably one from the other. Rabbit 1, Text-fig. 8, received a small dose of a virulent strain and after a severe and prolonged disease course showed a high concentration of serum immune substances.⁴ Rabbit 2 after a much larger dose of a pneumococcus of considerably less virulence, gave scarcely any evidence of disease but showed a well defined though low titer of pneumococcal promoting power in the serum. With



TEXT-FIG. 9. Rabbit 2. Experimental pneumococcus infection following the intraperitoneal injection of 0.001 cc. broth culture, *Pneumococcus* Type II. Reinoculation after recovery with 1 cc. broth culture.

Rabbit 3, on the other hand, the injection of a minute number of highly virulent pneumococci, produced no appreciable effect and no resulting detectable serum immunity but the animal was found to have developed a very greatly increased resistance. While Rabbit 2 with demonstrable serum immune properties showed no reaction

⁴ Further observations were prevented by the development of snuffles which progressed slowly to a fatal termination.



TEXT-FIG. 10. Rabbit 3. Experimental pneumococcus infection following the intraperitoneal injections of 0.000,000,01 cc. broth culture, Pneumococcus Type I, on December 4, and 0.1 cc. on December 31. Reinjection of 0.1 cc. on January 25.

following the injection of many times the lethal dose of culture, Rabbit 3, lacking such serum properties, developed a severe though not fatal disease after reinoculation. The subsequent appearance of immune substances in the serum of Rabbit 3 indicated a further increase in resistance as revealed by the lack of any detectable reaction to a second reinoculation of the same size.

Observations on the leucocyte count were not made in the rabbits chiefly on account of the uncertainty of the outcome of the experimental disease. Neither were the rabbits retested for duration of their active or passive immunity.

DISCUSSION.

The most noteworthy result of this work is the constancy of the findings. In the resistant animal recovery was always marked by the development of well defined humoral immune properties which failed to occur in fatal infections. In the susceptible animal studied (the rabbit) similar immune reactions were found although they could not be related so closely to the period of recovery. The observations in cats tend to amplify the studies of certain previous investigations on experimental pneumococcus infection in resistant animals. Bull (4), who has made the most detailed report on this subject, was not able to demonstrate agglutinins and opsonins in the serum of dogs until 24 to 48 hours after the temperature had fallen, but he found indirect evidence of immune body action *in vivo*, at the time of crisis. Whether the success obtained in our experiments in demonstrating the coincidence of the appearance of serum immune substances with recovery was due entirely to the methods employed or to the particular experimental animal used remains to be determined.

Our findings in the rabbit do not agree altogether with those of other investigators. The results obtained in the study of Rabbit 3, Text-fig. 10, offer a possible explanation for at least some of these previously observed irregularities in the appearance of immune substances in susceptible animals. This animal, as a result of an exceedingly small injection of a highly virulent pneumococcus developed a marked degree of active immunity but showed no evidence of passive immune properties in its serum. Is this to be attributed to the presence of immune substances in such low concentration that they could

not be detected in the test-tube or mouse, or were the body cells only sensitized and ready to react more energetically to further antigenic stimulus, or was this state of resistance due to another factor? The finding of a relatively high concentration of serum immune substances following the severe infection produced by the second inoculation suggests the first or second possibility. Another factor which may introduce variations in the demonstrability of humoral immune bodies is possible differences among the susceptible animal species in respect to excess elaboration of antipneumococcus substances. Cecil and Blake (15), for example, found only very occasionally slight traces of immune properties in the serum of monkeys tested 2 to 3 weeks following experimental pneumococcus pneumonia with all four types.⁵ It seems not improbable that certain non-resistant species, as the monkey, may elaborate specific antibodies in only slight excess of the concentration needed to combat the invading microorganism and that this detectable excess disappears rapidly after recovery. Dochez (16) observed in certain cases the rapid disappearance of protective substances from the serum of patients after recovery from lobar pneumonia. We have also noted, following lobar pneumonia, the complete disappearance within 10 days of serum immune substances which were present at the time of crisis in considerable concentration.⁶

The chief significance of our observations on the occurrence of antipneumococcus immune substances in the cat would seem to lie in the time relation of their appearance to the termination of infection and the nature of their demonstrable action. The fact that substances capable of promoting a marked degree of pneumococcal action appeared in the serum coincidentally with the amelioration of the disease process as shown by drop in temperature, and cessation of blood invasion which was followed by continued progress to recovery, makes it seem probable that the development of humoral immunity bears a causal relationship to the mechanism of increased antipneumococcus defense. A perfectly valid criticism of this assumption is that we have tested only the animals' serum and that we have no

⁵ That the monkey shows a well marked antibody production under certain conditions was found by Cecil and Blake (15) in their studies in vaccination with virulent living pneumococci.

⁶ This finding will be reported in a subsequent study on lobar pneumonia.

evidence that the serum of the recovering animal promotes the killing of pneumococci in the cat's body as it does in the test-tube with normal leucocytes. It is true that we have made no observations on the phagocytic and intracellular digestive activity of the leucocytes during experimental pneumococcus disease. The studies of other workers (17, 18, 19), however, on the activity of the leucocytes during pneumonia, while not in entire agreement with each other, indicate that ordinarily their function is little if any diminished, but in infections of marked severity there may be some depression especially of the intracellular digestive power. This phase of the problem requires further investigation.

The essential change which occurs in the serum of the cat recovering from pneumococcus infection would seem to be the elaboration of a heat-stable body or bodies whose chief antibacterial manifestations are opsonic and agglutinative activity. Power to precipitate the soluble substance undoubtedly is also present since the immune change is specific. That all these reactions are brought to bear on the pneumococcus growing in the animal body seems probable. Whether these several functions of the immune serum assume the same relative importance in the test-tube serum-leucocyte mixture is open to question, since conditions of pneumococcus growth *in vivo* differ in some respects from those *in vitro*. Possibly the precipitative action plays a less important rôle in the serum-leucocyte tests than in the body where the products of pneumococcus growth are present in greater concentration. Again, agglutination may play a more prominent part *in vivo*, especially in freeing the blood stream from organisms. However, the fact that the potency of an immune serum to produce pneumococcus killing in the serum-leucocyte mixtures parallels very closely its protective power in the mouse's body (14), suggests that its chief function is the same in both environments. Since phagocytosis and intracellular digestion are promoted to a marked degree by the presence of antipneumococcus serum and constitutes the only means of pneumococcus killing by the blood elements which we have been able to demonstrate, we are lead to infer that opsonic action in its entirety probably constitutes the most important property of such a serum.

The question then arises whether granting the possibility of de-

struction of pneumococci in the body by the above indicated means, will account for the degree of pneumococcus killing that occurs during recovery from infection. This question cannot be answered definitely since we have no means of estimating the number of organisms in the infected animal, but it is possible to estimate in a general quantitative way the killing power of immune blood. As shown in Table I, the immune horse serum is ten times as potent as the recovered cat serum. By repeated tests we have found that a 1 to 500 dilution of immune serum of this potency when added to the serum and leucocytes contained in 0.5 cc. normal blood is capable of causing the destruction of 10^{-3} of the standard pneumococcus suspension which amount contains approximately 1,000,000 pneumococci (14). With a serum one-tenth this potency we might expect that it would produce the same action in a dilution of 1 to 50, since the action of immune serum under these conditions has been found to be quantitative in nature. A cat weighing 2000 gm. would have a total blood volume of about 100 cc. and if each 0.5 cc. of blood could destroy 1,000,000 pneumococci the total amount disposed of by 100 cc. would be 200,000,000. But in making this estimate we are limiting the destruction to the normal number of circulating leucocytes. The serum is capable of sensitizing perhaps ten times or more as many organisms as can be cared for by the normal number of circulating white blood cells and it is likely, as shown by the work of others which will be discussed further on, that certain of the fixed tissue cells also take part in the phagocytosis under these conditions. Even if these figures, which are only approximate, err considerably on the side of too high an estimate they do show a relatively enormous killing potentiality in the serum and cells of the pneumococcus immune animal, and it is probable that this process occurs much more effectively and continuously in the body than in the test-tube.

The relation of the naturally immune state to these changes which have been found to take place during infection in the cat, is by no means clear. Assuming that the normal resistance of this animal depends chiefly on the presence of circulating antipneumococcus opsonins, it would seem probable that a pneumococcus sufficiently virulent to establish itself in a free cavity of the body must be able to

neutralize the normal immune substances.⁷ What happens next we can only surmise. This process of neutralization may take place rather slowly or it may be that the body has reserves of normal defense forces that can be called into action to retard the growth of the pneumococci until the development of the acquired immune reaction occurs. Wright considers that increased resistance may begin within a few hours after the introduction of the microorganisms and we also have reason to believe that the processes of acquired immunity are initiated relatively early in the disease course. The newly operating anti-pneumococcus opsonins detectable at recovery appear to differ in nature from the normal opsonins but we have as yet insufficient criteria to be sure of this. Our observations in a previous paper (3) on the relative heat resistance of normal pig opsonins raises the question as to the safety of relying entirely on this test for the differentiation of immune from normal opsonins. At any rate the pneumococcal action induced by the recovering cat serum in a rabbit serum-leucocyte mixture appears to be the same as that caused by the serum and leucocytes of the normal cat, but whether the pneumococcus-killing process which takes place in the animal successfully resisting infection by implanted pneumococci is the same as that employed by the diseased animal is open to question. Information concerning the type of cell engaged in phagocytosis and intracellular digestion of pneumococci in naturally resistant animals is far from complete. The evidence at hand, however, suggests that of the circulating leucocytes, the polymorphonuclears play the chief rôle in this process. The large monocytes have also been shown to be actively phagocytic for pneumococci. Observations in animals with acquired immunity indicate that a variety of cells take part in the disposal of pneumococcus.

Wright, studying the fate of pneumococci injected into rabbits immunized against *Pneumococcus* Type I found that in the lung the monocytes were fully as actively phagocytic as the polymorphonuclear leucocytes and that in the liver certain of the fixed tissue cells of the reticulo-endothelium engulfed large numbers of organisms. Singer

⁷ Bull (4) regards the early stages of disease as a period of adaption of the microorganism to the existing antibodies rather than to the exhaustion of these substances.

and Adler ascribe phagocytosis in both the immune and normal animal entirely to the reticulo-endothelium. However, they did not demonstrate this type of phagocytosis as occurring in the normal animal. Tudoranu found that in the aleuronat peritoneal exudate of rabbits immunized with *Pneumococcus* Type III the homologous organisms were phagocytosed almost entirely by the monocytes. The experiments of Winternitz and Kline (20) and also Wright in which the number of leucocytes were greatly reduced by benzene injections without appreciably diminishing the blood-clearing power of the immune animal, indicate that cells other than the leucocytes are active in the pneumococcus-destroying processes of the immune body. While all these experimental observations have been made in a susceptible animal, the rabbit, the same process may well occur in relatively resistant animals with acquired immunity and, were it possible to make observations in the normal animal body as easily as in the immune animal, cells, other than the leucocytes, might be found to be active in the destruction of pneumococcus. That the Kupffer cells of the liver in normal resistant animals are capable of taking up pneumococci seems quite probable but it is difficult to estimate the importance of this process in the normal defense mechanism against natural infection since we know practically nothing of the penetration of pneumococci into the blood stream except during disease.⁸

The observations on rabbits recorded in this study provide by themselves only indirect evidence as to the part played by humoral immunity in the rabbit's recovery from pneumococcus infection. But taken in conjunction with the findings in cats, they assume much greater significance. However, the fact should not be overlooked that acquired resistance in this animal has been shown to be present in the absence of demonstrable serum immunity and although certain plausible explanations for such absence can be made, there exists the possibility of other unknown immune factors.

It should be stressed that our experiments both with normal and infected animals have been carried on almost entirely with pneumococci of Types I and II. That the blood of pneumococcus-resistant

⁸ Kyes demonstration of the marked phagocytic power of the Kupffer cells of the pigeon for pneumococci suggests that these cells in the normal mammal may possess a similar function.

animals possesses the power to destroy *Pneumococcus* Type III (1) and that it possesses normal opsonins against this type (21), has been shown. But no experiments on infection with Type III pneumococci nor studies on normal resistance of susceptible animals against these organisms have been made. Tillett (22) has recently published a study which might be interpreted to indicate that natural and acquired immunity to *Pneumococcus* Type III may be of a different order than that against the other two fixed types. He found that the resistance of normal rabbits to relatively enormous intravenous doses of an encapsulated Type III pneumococcus was not associated with demonstrable antibodies in the serum and that little evidence of phagocytosis could be detected in the blood. Phagocytosis by the reticulo-endothelial system was not investigated. The most significant phase of Tillett's work deals with the production of active immunity to Type III which he finds to be not only not associated with demonstrable immune substances but to be non-specific. He considers that the normal rabbit possesses a mechanism for inflicting injury on the capsule of the Type III pneumococcus and that active immunity probably consists in an increase in this normal function.

In the light of our present incomplete knowledge it is not possible to draw any general conclusions concerning the processes underlying recovery from experimental pneumococcus infection. Until the whole field has been studied with the care that has been devoted to certain areas, the interrelationship of the component parts will probably not be revealed. However, the constant occurrence of phenomena observed in the study of certain aspects of the problem justify our drawing tentative inferences limited always to the conditions under which the observations were made. Thus the evidence at hand would indicate that recovery from experimental infection with *Pneumococcus* Types I and II in cats and rabbits is brought about largely by the elaboration of specific antipneumococcus serum substances whose chief function is to make possible phagocytosis and intracellular digestion of the invading pneumococci. That the evidence for this assumption is much clearer in cats than in rabbits is granted. Furthermore drawing the above conclusion does not exclude the possibility of other forces aiding in the process. There may be another factor in acquired immunity and certain experimental findings suggest its

existence, which plays a part perhaps relatively more important in one type of animal than another and possibly varying in its activity with the different types of pneumococci. That other pneumococcus-resistant animals react in a manner similar to the cat seems probable in view of observations of other investigators and because of the fact that these animals show the same type of normal protective mechanism. Inferences concerning the recovery processes of pneumococcus-susceptible animals other than the rabbit are less safe because of the great diversity of findings by different workers. However, the fact that humoral immune substances have been detected in these animals with acquired resistance, suggests that this form of immunity plays some part in their recovery from pneumococcus infection.

SUMMARY.

A study was made of the blood of cats and rabbits during experimental pneumococcus infection with a view to ascertaining the relationship of acquired immune properties to the mechanism of recovery. Observations were directed chiefly towards the detection of pneumococcal promoting substances, but the other manifestations of anti-pneumococcus reaction were studied as well. It was found constantly that the serum of animals recovering from infection possessed the power to promote the destruction of highly virulent pneumococci in rabbit serum-leucocyte mixtures which mixtures of themselves have no growth inhibitory action. Furthermore, the presence of this serum immunity was associated with a marked increase in acquired resistance to the pneumococcus. In cats which were studied in the most detail the pneumococcal promoting power of the serum as well as the opsonic, agglutinative, and mouse protective activities became demonstrable at the time of recovery and their appearance in the serum always marked the termination of blood invasion. These immune reactions were found to be type-specific. The animals which succumbed failed to develop detectable serum immune properties and showed persistent blood invasion. The degree of leucocytosis did not appear to bear any constant relation to the outcome of the disease. The significance of these findings is discussed.

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Edited by

W. J. CROZIER

JOHN H. NORTHROP

W. J. V. OSTERHOUT

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SOME FACTORS DETERMINING THE LOCALIZATION OF A CHICKEN TUMOR AGENT.*

BY RONALD D. MACKENZIE, M.B.,† AND ERNEST STURM.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

PLATES 19 AND 20.

(Received for publication, October 4, 1927.)

In the production of many pathological processes, two main factors are concerned. The first, the general factor, which determines the nature of the condition, and the second, or local factor, determining the site of the inflammatory process.

In osteomyelitis for example, there is frequently the history of an antecedent injury to the affected part, and it may be taken for granted, that this injury in some way predisposes to a localization of bacteria, which may happen to be present in the circulating blood or elsewhere. In syphilis, tertiary lesions appear more frequently in superficial bones which are more exposed to injury than in the deeper ones which are well protected by overlying tissues. Kiedel and Zimmermann (1) have described an interesting case, showing that under certain conditions a syphilitic eruption may follow the lines of tattoo markings in the skin, and further, that certain of the pigments used in tattooing are more active than others in this relation, though whether because of a specific action of the dye substance, or of the degree of reaction that it induces is not known. That all inflammatory reactions are not equally effective in promoting the growth of organisms, is well shown by the work of Chesney and Kemp (2), who have demonstrated, that spirochetes will survive better and multiply more rapidly in a recent granulating wound, than in an older subacute lesion.

Various attempts have been made to study the question of localization experimentally.

Thus Gye and Kettle (3) have demonstrated the localizing effect of silica in tuberculosis. Levaditi and Nicolau (4) have shown that the trauma produced by the removal of hair is sufficient to determine a localization of the eruption,

* This investigation was carried out by means of funds from the Rutherford Donation.

† Fellow of the Rockefeller Foundation.

when vaccine virus is injected intravenously. Somewhat similar results may be obtained when the "virus" of epithelioma contagiosum is injected intravenously in birds, the lesions developing in this case around the roots of feathers which have recently been plucked (5).

Rous and Murphy and Tytler (6) while working with the filtrates of those chicken tumors, which can be transmitted by means of a cell-free filtrate, found that a larger number of tumors could be obtained, if a certain amount of tissue derangement was caused at the site of the injection. To produce this they added kieselguhr to the inoculum. This substance has been shown by Podwysoski (7) to produce a well marked tissue reaction when injected subcutaneously. Jones and Rous found that injury determined the localization of the growths developing after the inoculation of mouse tumor material into the peritoneal cavity (8).

Metastasis formation by "filterable chicken tumors" is common, probably because, as shown by Rous, Robertson and Oliver (9), the active agent is frequently present in the blood of chickens with developing tumors. The secondary growths may occur in the liver, lungs, etc., but during the spring season they are much more common in the ovary. It has been suggested, that this seasonal infection of the ovary is due to the frequent injuries received by that organ during the process of ovulation, and the resulting presence of much recent granulation tissue, which it seems is readily attacked by the tumor agent. Rous, Murphy and Tytler (6) investigated the action of the tumor agent when injected intravenously in chickens, and found that tumors seldom resulted, but when they did develop it was nearly always in the functioning ovary.

The above observations on chicken tumors suggested that the induced inflammatory reactions might prove favorable to the localization of the causative agents on intravenous injection with as a result the subsequent development of tumors. It was therefore determined to attempt the experiment.

Method.

The method selected was the injection into the pectoral muscles of various irritating substances such as Scharlach R, kieselguhr, tar and hashed chicken embryo tissue, followed after a period by the intravenous injection of fresh tumor filtrate. The pectoral region was selected as the site, partly for convenience, and partly because metastases have never been reported there, even after intravenous injection of the tumor agent. Chicken Sarcoma 1 of the series described from The Rockefeller Institute was chosen for use in these experiments, and only adult Plymouth Rock and Rhode Island red hens were used. These birds are now equally susceptible to this tumor.

Experiments with Scharlach R.

First Experiment.—Six hens received 1 cc. of a saturated solution of Scharlach R in olive oil in each breast and this injection was repeated after 7 days. There resulted a certain amount of inflammatory reaction which could be made out on palpation. Three weeks after the last injection of Scharlach R each fowl received 5 cc. of fresh tumor filtrate into the wing vein. This filtrate was prepared in the usual way by grinding about 25 gm. of the tumor material with sand and about 400 cc. of Ringer's solution, centrifuging to remove the larger particles and filtering through a Berkefeld V filter.

Four weeks later the fowls were killed and thoroughly examined. The pectoral muscles showed a marked inflammatory reaction around the Scharlach R which had become localized and was lying in pockets shut off by well formed connective tissue. Only in one bird out of the five was a tumor present in the breast tissue, this being in contact with the reaction tissue. That this failure to localize in the pectoral tissue was not due to general resistance of the chickens or to the low potency of the filtrate was clearly demonstrated by the fact that four of the five animals showed formations of tumors in other locations, namely lungs, spleen, ovaries, liver and in the wing at the site of the filtrate injection.

In only one fowl out of six employed in this experiment a tumor developed in the breast muscle in the region infiltrated with Scharlach R. In view of this fact the procedure was slightly altered, the period between the second injection of Scharlach R and the intravenous injection of tissue filtrate being shortened.

Second Experiment.—Five hens were injected into the breast muscle with the same solution of Scharlach R as before. Four of these received 1 cc. on each side, while the last received only 0.5 cc. After an interval of 7 days these injections were repeated. A week later, the intravenous injection of 5 cc. of fresh tumor filtrate prepared as described above was made into a wing vein. The animals were killed 4 weeks later, and the breast tissue was examined.

As before, the solution of Scharlach R spread through the pectoral tissue and became only slightly encapsulated by fine connective tissue. In all these birds, small tumor nodules were found in the breast muscles, lying amongst the Scharlach R (Fig. 1). This was later confirmed by microscopical examination. The smaller amount of the dye received by one of the hens did not appear to affect the result, which was the same in all the injected fowls.

Experiments with Tar.

Following the same plan as before, three fowls were injected with 1 cc. of a solution of purified tar in benzene. This was repeated after a week. Seven days later they were given an intravenous injection of 5 cc. of tumor filtrate. When the birds were examined at a later date, they all showed tumor formation in the breast muscles in the region of the tar injections (Fig. 2). Evidently tar resembles Scharlach R in its localizing effect.

Experiments with Kieselguhr.

The reaction produced by kieselguhr in the tissues is a peculiar one, and has been studied by Podwyssozki (7) and others. It consists very largely of giant cells, probably of the foreign body type, with little of the ordinary connective tissue (Fig. 3). It seemed of interest to attempt localization with this substance. Accordingly five hens were inoculated with a mixture of kieselguhr in Ringer's solution, 1 cc. being injected into each breast. This was repeated in a week, and then 7 days after the second injection, the tumor filtrate was passed into a wing vein. One of the birds died early in the experiment and was discarded. Of the remainder, only one out of the four developed a tumor of the breast muscle. All showed tumor localizations in other parts of the body.

This experiment was carried out under conditions very similar to those of the second experiment with Scharlach R and the one with tar. The same filtrate was used for the three. The differing results would suggest that the nature of the tissue reaction is of importance in determining the degree of localization.

Experiments with Embryonic Tissue.

In these experiments the local change was induced with fresh chick embryo tissue.

First Experiment.—7 to 10 day embryos were hashed up in Ringer's solution, and 1 cc. injected into the breast muscle of each of five hens. Only one injection of embryo tissue was given to each bird. When, after 10 days, well formed embryomas had appeared, an injection of 5 cc. of fresh tumor filtrate was given into a wing vein. After 21 days had elapsed, a time presumably sufficient for localiza-

tion to have taken place in or around the embryo nodules, the birds were killed and examined. Of the five used, only one, showed such a localization of the growth. That the filtrate was active and the birds not resistant was shown by the fact that all five developed tumors in other regions. Furthermore the filtrate was the same as that used for the second Scharlach R experiment where localization took place in the areas of induced reaction in all the fowls injected.

In view of the results obtained in the second Scharlach R experiment, it was decided to reduce the time between the injection of embryo tissue and the injection of the filtrate.

Second Experiment.—Four hens were injected into the pectoral muscles with 1 cc. of tissue. Two of these were then injected intravenously with 5 cc. of tumor filtrate 3 days after receiving the embryo tissue, and the other two received their injections 5 days after the embryo hash. The results were as follows:

(a) The chickens injected with filtrate 3 days after the introduction of the embryonic tissues had numerous tumor nodules in and around the embryoma (Fig. 4), all being in contact with the embryonic tissue. These findings were verified by microscopic examination.

(b) The chickens which received the filtrate 5 days after the embryonic tissue injection showed macroscopically no evidence of tumor localization but microscopic examination disclosed in one fowl a few scattered areas of sarcomatous transformation in the embryonic tissue.

The same filtrate was used in both of these groups and its potency was shown by the fact that all of the fowls developed tumors in other parts of the body.

While the first and second experiments were carried out with different filtrates it seems justifiable to compare the results, since in both the filtrate was very active as shown by the fact that all of the fowls developed tumors elsewhere than in the breast. The findings suggest that the reaction induced by the injection of embryo tissue is only active as a localizing factor in its early stages.

DISCUSSION.

Localization of the agent of Chicken Tumor 1, resulting in tumor formation has been accomplished by inducing the development of reaction tissue in the pectoral muscles prior to the intravenous injections of fresh tumor filtrate.

The irritants successfully used for the purpose differ somewhat in their effect, those in one group, Scharlach R, tar and embryo tissue,

eliciting an ordinary subacute reaction of the fibroblastic type, while kieselguhr gives rise to the development of a peculiar tissue consisting largely of giant cells. In the experiment with irritants of the first type, the tumor localization occurred most frequently in the early stages of the reaction when the reaction cells were young and actively growing. As the reaction tissue became older less localization in it took place.

While the number of fowls injected with kieselguhr was relatively few, the small proportion of positive results obtained with this substance has some significance, since the filtrate employed was the same which caused tumors in all of the fowls that had been injected with Scharlach R and tar. It may be concluded tentatively that the peculiar reaction tissue elicited by kieselguhr is less favorable to the localization of the tumor-producing agent than is that induced by other substances. It is of interest to note that kieselguhr is less active in localizing vaccine virus after intravenous injection than is the case with a variety of other substances (10).

Earlier work by Murphy and Rous (11) has shown that in order to produce Chicken Tumor 1 in the embryo the agent must be brought into contact with mesodermal tissue. It is reasonable to suppose that young and actively growing connective tissue will be more susceptible than older tissues to the influence of the tumor agent. This supposition is supported by the above experiments. They would also suggest that it is the reaction to injury rather than the injury as such which renders a tissue susceptible to the tumor-producing activity of the agent.

SUMMARY.

A localization out of the blood stream of the agent causing a chicken tumor, with the subsequent development of the growth can be brought about in the breast muscle, by inducing in this tissue a reaction by such substances as Scharlach R, tar, embryonic tissue and kieselguhr. Localization in the reaction tissue elicited by kieselguhr takes place relatively infrequently. The earlier stages of the reaction induced by these substances localize the tumor agent more regularly than the later stages of the reaction.

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EXPLANATION OF PLATES.

PLATE 19.

FIG. 1. Localization of Chicken Tumor 1 in a region injected with Scharlach R in olive oil. Clear spaces which contained the Scharlach R are surrounded by young connective tissue, and lying alongside is a mass of tumor tissue.

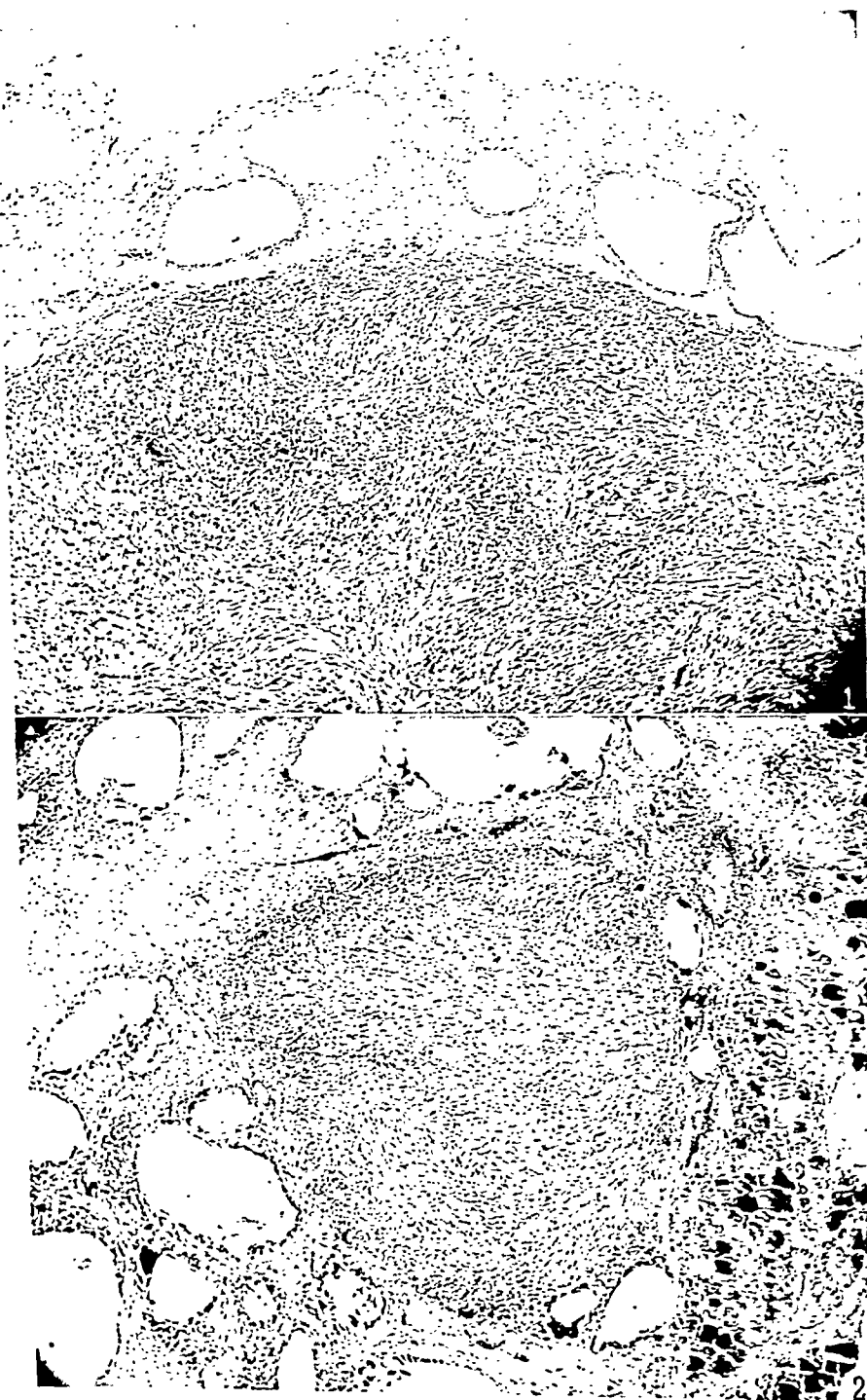
FIG. 2. Localization of chicken tumor in a region injected with tar. Clear spaces indicate position of tar, and in between these is much young connective tissue. In the center is the tumor nodule. The dark spots are more or less damaged muscle fibers.

PLATE 20.

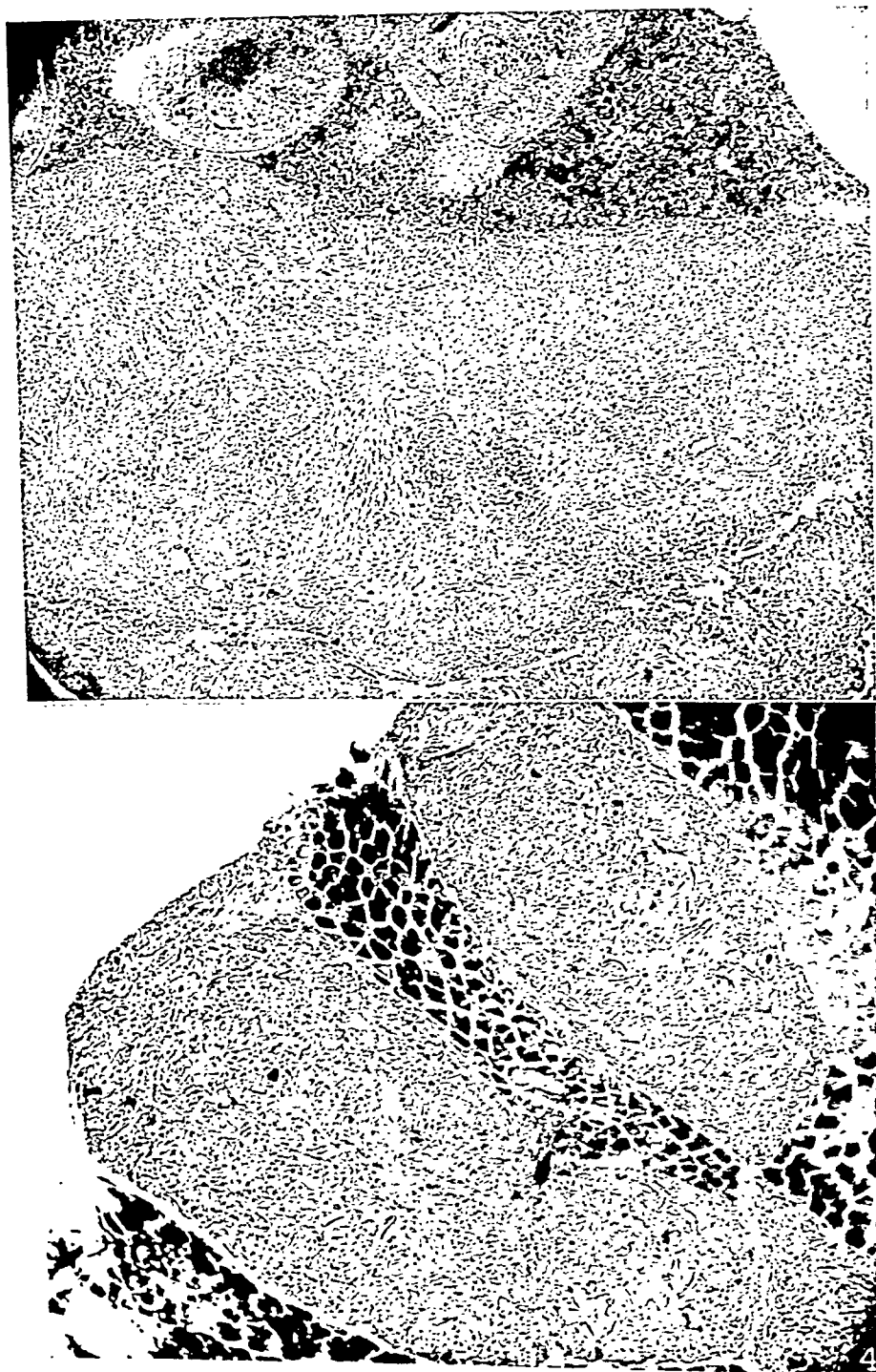
FIG. 3. Area of kieselguhr reaction in chicken muscle. This is composed mainly of giant cells with very little connective tissue. There is no localization of the tumor agent.

FIG. 4. Localization in a region of embryoma formation. A well formed tumor nodule is seen above, and below is a mixture of embryonic tissues, composing the embryoma.





(Mackenzie and Sturm: Localization of chicken tumor agent.)



(Mackenzie and Sturm: Localization of chicken tumor agent.)

THE EFFECT OF DIGESTS OF PURE PROTEINS ON CELL PROLIFERATION.

BY LILLIAN E. BAKER, PH.D., AND ALEXIS CARREL, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

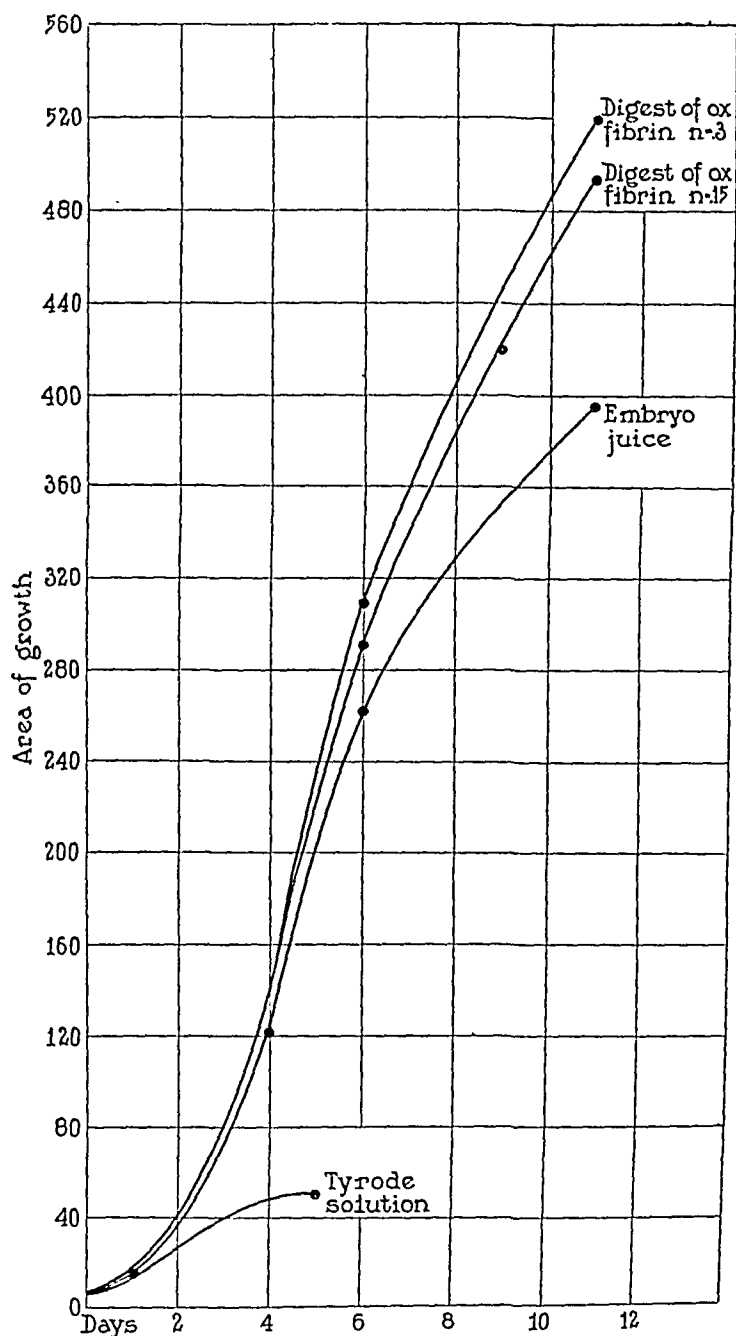
(Received for publication, December 2, 1927.)

It has been reported that fibroblasts, epithelial cells, and leucocytes display great proliferative activity when cultivated in the peptic digestion products of proteins.¹ That statement was based on experiments with digests of complex substances, such as embryo pulp, egg white, commercial ox fibrin, and Witte's peptone. In order to ascertain conclusively whether cell multiplication was due to the hydrolytic products of the proteins themselves, or to other substances in the material used, the experiments were repeated with proteins which were purified before being hydrolyzed. For this purpose, fibrin, egg albumin, and edestin were used, because the digestion products of commercial fibrin have been found to possess a greater growth-promoting effect than any other substance, and because egg albumin and edestin may be purified by crystallization.

Effect of Split Products of Fibrin on Cell Proliferation.

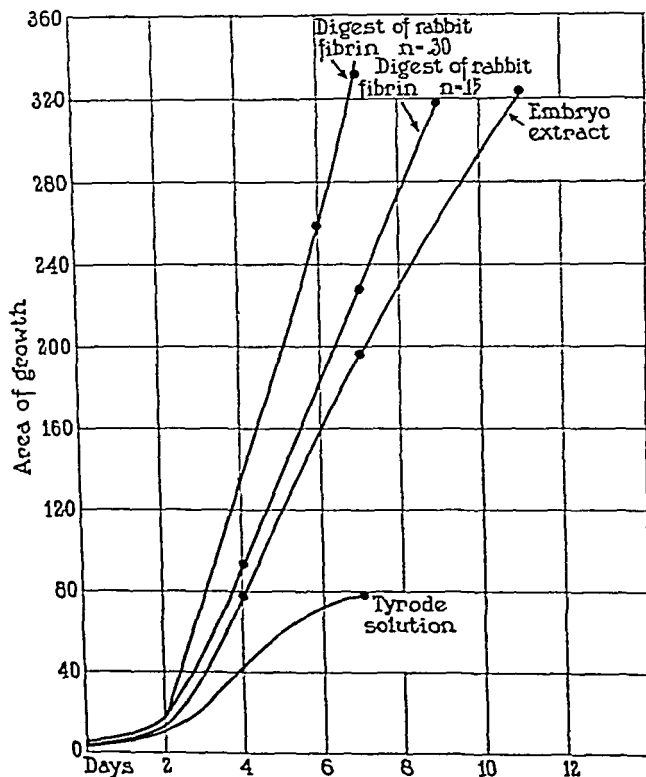
The fibrin was prepared from both ox and rabbit blood. The ox blood was citrated by drawing it into a 2.5 per cent sodium citrate solution in 0.9 per cent salt solution, using 100 cc. per liter of blood. The rabbit blood was taken in paraffined tubes and kept in ice to prevent coagulation. After centrifugation of the blood, the upper layers only were drawn off and again centrifuged in order to eliminate all the corpuscles from the plasma. The fibrin was precipitated by half saturation with sodium chloride and washed three times with a half saturated salt solution, three times with 0.9 per cent sodium chloride, and then with large volumes of water. The dry fibrin was digested for 24 hours at 40°C., 2.5 gm. of fibrin being suspended in 40 cc. of 0.5 per cent pepsin in N/20 HCl. Since some fibrin remained undigested, a large proportion of the primary split products was obtained in this way. The solutions were neutralized with N/1 NaOH,

¹ Carrel, A., and Baker, L. E., *J. Exp. Med.*, 1926, xliv, 503.



TEXT-FIG. 1. Experiment 9162 D. Comparison of the growth of fibroblasts from fresh embryo heart in a digest of purified ox fibrin, with that in embryo juice and Tyrode solution.

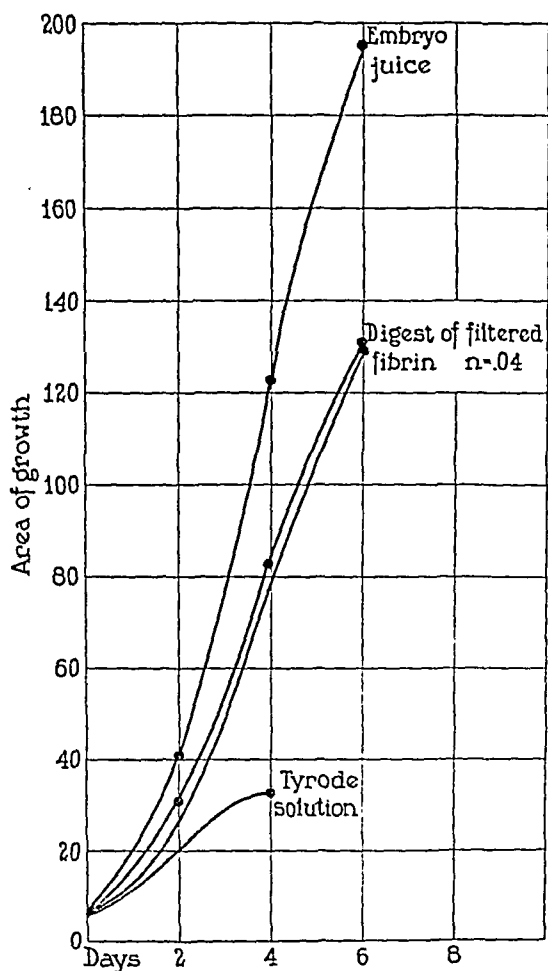
boiled to one-half volume in order to remove the coagulable substances and expel the toluene which was used as a preservative. After determination of the cryoscopic point, the solution was brought to isotonicity and a pH of 7.4. These digests were tested at various concentrations on fibroblasts from embryonic



TEXT-FIG. 2. Experiment 3606 A. Comparison of the growth of fibroblasts from fresh embryo heart in a digest of purified rabbit fibrin, with that in embryo juice and Tyrode solution.

heart cultivated in flasks according to the usual method, the fluid being placed on the surface of a coagulum of diluted plasma. Every 48 hours, the fluid was removed, the culture washed with Tyrode solution, and fresh fluid supplied. The area of the colony was traced under a projectoscope and measured with a planimeter.

The digest of purified ox fibrin, and also that of rabbit fibrin, at an N concentration of from 0.15 to 0.3, caused a large growth of fibroblasts from embryonic heart, as large or larger than that which occurred in



TEXT-FIG. 3. Experiment 9225 D. Comparison of the growth of fibroblasts from fresh embryo heart in a digest of ox fibrin from filtered plasma, with that in embryo juice and Tyrode solution. The concentration of nitrogen in this digest was much less than in the previous ones.

embryo juice (Text-figs. 1, 2; Table I). The results were entirely comparable with those obtained when commercial fibrin was used. It is evident, therefore, that the substances which cause this rapid

proliferation of cells are the hydrolytic products of fibrin and not the impurities contained in commercial fibrin such as blood corpuscles. One sample of ox plasma was filtered through a Berkefeld filter. As the filter soon became clogged, only a small amount of fibrin was obtained. The quantity was so small that only a dilute solution of the digest could be used in the culture medium at a nitrogen concentration of 0.04 per cent. Nevertheless, a considerable growth of fibroblasts occurred (Text-fig. 3).

Effect of Crystalline Egg Albumin Digests on Cell Proliferation.

The egg albumin was purified by three crystallizations with ammonium sulfate. To remove the ammonium sulfate which previous experience had shown to be toxic even after long dialysis of the albumin, two methods were used. In the first, the crystals were washed three times for 24 hours with a solution of saturated sodium chloride containing 1 per cent acetic acid. This denatured part of the albumin. The second method consisted of recrystallization three times with a mixture of sodium and potassium sulfates, making a total of six crystallizations. The albumin was finally dialyzed in very permeable collodion bags for 7 days in running tap water and 3 days in distilled water.

Several digests were made of this albumin, the quantity of pepsin, the time of digestion, and the concentration of the solution being varied. Some crystalline albumin was coagulated by heat before digestion and the process interrupted as soon as the albumin was transformed into soluble material. A large proportion of primary split products was thus secured. The methods of preparing the digests are summarized in Table II. In all cases, the solutions were boiled and prepared in the same manner as the fibrin digests. These digests proved toxic at nitrogen concentrations as high as those used for the fibrin digests and therefore were diluted with Tyrode solution to a nitrogen concentration of 0.17 to 0.07 per cent before being used as a culture medium. They were tested in the manner outlined above on fibroblasts growing from embryonic heart. The increased area of growth of fibroblasts from fresh heart in the albumin digests over that of control tissues in Tyrode solution varied from 75 to 400 per cent, according to the digest used (Table III). Some of the best results were obtained with digests prepared from heat denatured albumin. In order to ascertain whether this previous denaturation influenced

the nutritive value of the digests, two preparations were made (x-512 and x-513) from the same solution, under identical conditions except that one (x-512) was first denatured by heating in N/100 HCl solution for 15 minutes at 60°C. No differences were found in their nutritive effect.

The digests of crystalline albumin did not, however, promote as large or rapid a growth of fibroblasts as is caused by the digests of fibrin or by embryo juice. The area of growth was about equal to, but not as dense as that in embryo juice diluted to 50 per cent of its usual concentration (Table IV). Moreover, the cells of the controls in the diluted embryo juice remained in normal condition, while those in the digests finally degenerated, an evidence that although the albumin split products cause growth, they are incomplete from the nutritive standpoint.

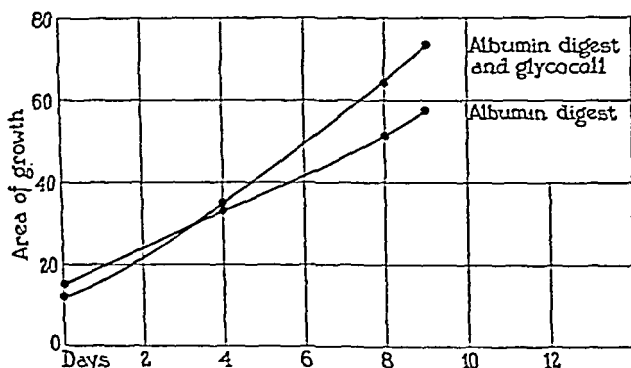
The deficiency of the egg albumin digests was still more evident when a 15 year old strain of chicken fibroblasts was used in the experiments. The growth in the albumin digests was very much smaller than that of the controls in embryo juice, and only slightly greater than that of the controls in Tyrode solution. In all cases, the growth ceased after a few days, the cells became filled with fat, and the plasma clot usually liquefied immediately around the tissue, making it impossible to secure accurate quantitative data. It is probable that the larger growth of the fibroblasts from embryonic heart is due to the presence in the fresh tissue of some substances which are needed to supplement the nutritive action of the digestion products of crystalline egg albumin.

Effects on Cell Proliferation of Digests of Crystalline Egg Albumin and Casein Supplemented by Glycocoll and Nucleic Acid.

It seemed probable that the great differences in the nutritive value of the digests of fibrin and of crystalline egg albumin might be due to the difference in their amino acid content. Since crystalline albumin contains no glycocoll, the addition of sufficient glycocoll² solution to bring its concentration up to that of the fibrin digests was tried, and

² The glycocoll solution contained 50 mg. of crystalline glycocoll in 100 cc. of Tyrode solution. It was sterilized by filtration through a Berkefeld filter.

also the addition of a digest of gelatin,³ a protein particularly rich in glycoll. For these experiments, a pure strain of sarcomatous fibroblasts of the rat was used, since it was the only pure strain of fibroblasts immediately available which did not cause liquefaction of the coagulated plasma. These cells grew very slightly in the digest of crystalline albumin, in fact, hardly any more than when cultivated in Tyrode solution. The addition of glycoll increased the rate of growth about 70 per cent (Text-fig. 4, Table V), and the gelatin digest about 61 per cent. Similar results were obtained when the experi-



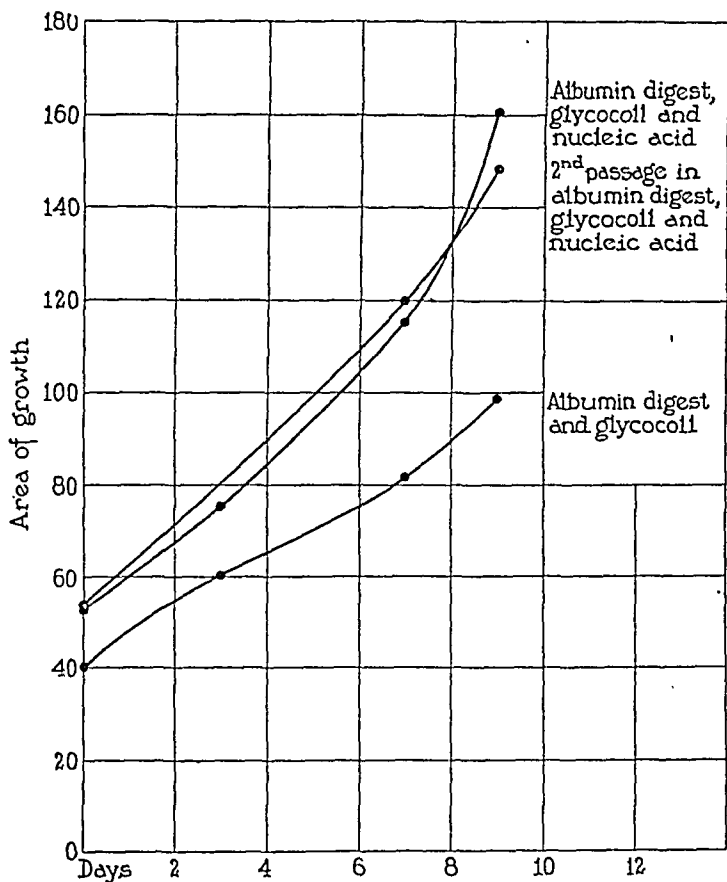
TEXT-FIG. 4. Experiment 6140 C. Effect of the addition of glycoll to a digest of crystalline egg albumin on the rate of growth of sarcomatous fibroblasts of the rat.

ments were repeated with digests of casein⁴ which contains only an exceedingly small quantity of glycoll (Table VI), showing beyond doubt that glycoll is an essential component of the culture medium,

³ The gelatin digest was prepared by incubating 5 gm. of gelatin with 1 gm. of pepsin in 200 cc. of $N/20$ HCl at 37°C. for 24 hours. It was neutralized, boiled, and adjusted for cultures as usual. It contained 0.45 per cent of nitrogen and 0.048 per cent of amino nitrogen. Before use on the cultures, it was diluted to 0.25 per cent of nitrogen.

⁴ The casein digest was prepared by incubating 10 gm. of casein at 37°C. for 24 hours, with 0.5 per cent of pepsin in 200 cc. of $N/20$ HCl. This was also neutralized, boiled, made isotonic, etc. It was diluted to 0.4 per cent of nitrogen before being used on the cultures. Its ratio of total to amino nitrogen was 7.2, and 67 per cent of its nitrogen was in the form of proteose.

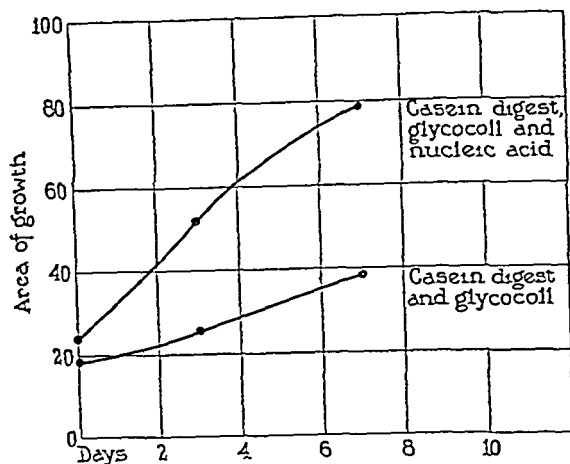
either as such, or in peptide form, and that one of the reasons for the different nutritive actions of protein digests lies in their varying amino acid content.



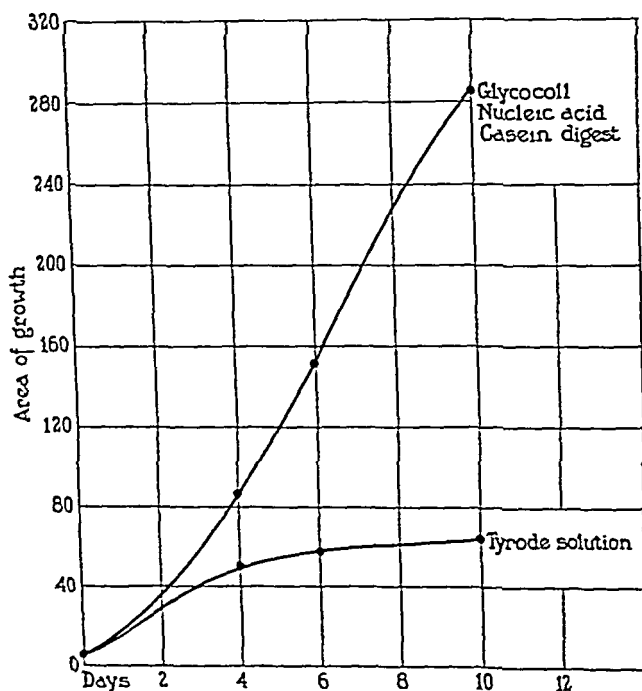
TEXT-FIG. 5. Experiment 6162 C. Effect of the addition of nucleic acid to a mixture of glycocoll and a digest of crystalline egg albumin on the rate of growth of sarcomatous fibroblasts of the rat. The tissue having the nucleic acid in its medium grew uniformly through two passages, while the control in glycocoll and albumin digest died at the end of the 1st passage.

However, even after the addition of glycocoll, the growth was small and the tissues survived only a few days longer than in Tyrode solution. Then, thymus nucleic acid⁵ was added to the medium. This

⁵ The thymus nucleic acid solution contained 0.1 gm. of nucleic acid in 100 cc. of Ringer's solution. N/1 NaOH was added to dissolve it and bring the pH to 7.4. It was sterilized by filtration through a Berkefeld filter.



TEXT-FIG. 6. Experiment 9454 D. Effect of the addition of nucleic acid to a mixture of glycoll and a digest of casein, on the rate of growth of sarcomatous fibroblasts of the rat.



TEXT-FIG. 7. Experiment 9504 D. Growth of a 15 year old strain of normal chicken fibroblasts in a mixture of glycoll, casein digest, and nucleic acid.

greatly improved its nutritive value (Text-fig. 5). An additional increase of approximately 97 per cent in the area of growth was obtained when nucleic acid was added to a mixture of crystalline egg albumin digest and glycocoll (Table VII), and an additional increase of approximately 193 per cent when it was added to a mixture of glycocoll and casein digest (Table VIII). In fact, the strain of malignant fibroblasts grows at a rapid rate in the very simple mixture of just three substances: digest of casein or crystalline egg albumin, glycocoll, and thymus nucleic acid dissolved in Tyrode solution. Growth in a mixture of the three substances was better than in any two of them (Tables IX and X). Not only did the presence of nucleic acid increase the rate of growth, but it also greatly lengthened the time of survival of the tissues. In the digest of crystalline egg albumin, glycocoll, and nucleic acid, colonies of the sarcomatous fibroblasts have grown actively for more than 20 days. In the mixture of glycocoll, nucleic acid, and casein digest, some of the cultures were still active after more than a month. The colonies have been divided several times, showing that a considerable increase in the mass of the tissues has occurred. Still more encouraging is the fact that the cells remained in normal condition throughout the experiment, although the rate of growth was much less than that of colonies cultivated in embryo juice. Many other substances have been added to this simple medium, such as iron, vitamins, salts, cystine, cholesterol, etc., but as yet no conclusive evidence of their nutritive value has been obtained.

Effect of Vegetable Protein Digests on Cell Multiplication.

Since both crystalline egg albumin and casein, which have been used in the synthetic media, are deficient in glycocoll experiments were undertaken with digests of edestin which is rich in glycocoll and can also be crystallized. Crystalline edestin obtained from Hoffman-La Roche was used. Digests of this protein⁶ were found to promote the proliferation of rat sarcomatous fibroblasts much more actively than either those of casein or crystalline egg albumin. It is

⁶ The edestin digest was prepared by digesting 2.5 gm. of crystalline edestin in 100 cc. of 0.5 per cent pepsin in N/20 HCl for 20 hours. It was also neutralized, boiled, and made isotonic.

TABLE I.

Comparison of the Rate of Growth of Fibroblasts from Embryo Heart in Embryo Juice and in a Digest of Purified Fibrin.

Experiment No.	Preparation No.	Nitrogen concentration	Time of growth	Area ⁷ of growth in embryo juice. Control	Area ⁷ of growth in fibrin digest. Experiment	Ratio: area of experiment to area of control	Remarks
		<i>per cent</i>	<i>days</i>				
6068 C 1-2	x-525	0.208	8	87	83	0.95	Ox fibrin
6017 C 1-3	x-525	0.208	7	72	126	1.75	" "
3606 A 1	x-468	0.30	7	200	356	1.78	Rabbit "
3606 A 2	x-468	0.15	7	200	240	1.20	" "
3606 A 3	x-468	0.30	7	200	194	0.97	" "
3606 A 4	x-468	0.15	7	200	170	0.85	" "
9162 D 1	x-467	0.15	10	370	460	1.24	Ox "
9162 D 2	x-467	0.30	10	370	480	1.30	" "
Average.....						1.26	

TABLE II.

Methods of Preparing Albumin Digests.

Preparation No.	Kind of albumin	Preliminary treatment	Volume of solution used	Pepsin	Concentration of HCl	Time of digestion	Ratio: total to amino nitrogen
			<i>cc.</i>	<i>per cent</i>		<i>hrs.</i>	
x-360	Crystallized 3 times. Washed, 1 per cent acetic in saturated NaCl.	Coagulated by heat	200	0.5	N/20	5	4.7
x-383	" "	Acid denatured	60	0.5	N/30	4	7.1
x-502	" "	" "	50	0.5	N/20	5	8.2
x-503	" "	" "	72	0.5	N/20	10	6.5
x-504	" "	Denatured by heat	72	0.5	N/20	5	7.1
x-512	Crystallized 6 times, 3 from Na ₂ SO ₄ and 3 from K ₂ SO ₄	" " "	80	0.5	N/20	15	6.5
x-513	" "	Not denatured	80	0.5	N/20	15	6.4
x-534 ⁸	" "	Coagulated by heat	200	0.25	N/20	7	6.7

⁷ This is the area in square centimeters of the projected image of the colony of cells, and is 62 times the size of the colony.

⁸ 72 per cent of the nitrogen of this preparation was precipitated by saturated sodium sulfate at 37°C. Therefore, it consisted largely of proteose.

TABLE III.

Effect of Digests of Crystalline Egg Albumin on the Rate of Growth of Fibroblasts from Embryonic Heart. Tyrode Solution Used as Control.

Experiment No.	Preparation No.	Ratio: total N to amino N	Nitrogen concentration	Time of growth	Area ² of growth in Tyrode solution. Control	Area ² of growth in albumin digest. Experiment	Ratio: increased area of experiment to control
				days			
8816 D	x-360	4.7	0.085	7	50	210	4.2
3922 C	x-360	4.7	0.085	8	90	170	1.9
3922 C	x-360	4.7	0.170	9	110	353	3.2
3353 A	x-360	4.7	0.085	8	110	170	1.5
3353 A	x-360	4.7	0.170	8	110	270	2.5
3961 C	x-360	4.7	0.170	10	70	140	2.0
3968 C	x-360	4.7	0.170	4	38	68	1.8
8818 D	x-360	4.7	0.170	5	49	95	1.9
3347 A	x-360	4.7	0.170	5	48	98	2.0
3991 C	x-383	7.1	0.120	7	55	268	4.9
9240 D	x-502	8.2	0.070	6	50	88	1.8
9240 D	x-503	6.5	0.070	6	50	95	1.9
9240 D	x-504	7.1	0.070	6	50	95	1.9
4855 C	x-513	6.4	0.120	9	10	47	4.7
4855 C	x-512	6.5	0.120	9	10	70	7.0
4909 C	x-512	6.5	0.120	7	15	61	4.0
4909 C	x-513	6.4	0.120	7	15	60	4.0
4828 C	x-512	6.5	0.120	9	10	46	4.6
4828 C	x-513	6.4	0.120	9	10	38	3.8
Average.....							3.1

TABLE IV.

Comparison of the Effect of Digests of Crystalline Egg Albumin and of Embryo Extract Diluted 50 Per Cent on the Rate of Growth of Fibroblasts from Fresh Embryo Heart.

Experiment No.	Preparation No.	Nitrogen concentration	Time of growth	Area ² of growth in embryo extract. Control	Area ² of growth in albumin digest. Experiment	Ratio: area of experiment to area of control	Remarks
		per cent	days				
4855 C	x-512	0.12	9	57	70	1.23	Thin growth in experiments
4828 C	x-512	0.12	9	45	46	1.02	" " " "
4909 C	x-512	0.12	7	52	61	1.17	" " " "
4854 C	x-512	0.12	10	28	32	1.14	" " " "
4855 C	x-513	0.12	9	57	47	0.82	" " " "
4828 C	x-513	0.12	9	45	38	0.84	" " " "
4909 C	x-513	0.12	7	52	60	1.15	" " " "
4854 C	x-513	0.12	10	28	29	1.03	" " " "
Average.....							1.05

TABLE V.

Effect of the Addition of Glycocoll to a Digest of Crystalline Egg Albumin⁹ on the Rate of Growth of Sarcomatous Fibroblasts of the Rat.

Experiment No.	Preparation No.	Nitrogen concentration	Time of growth	Area ⁷ of growth. Control ⁹	Area ⁷ of growth. Experiment ¹⁰	Ratio: area of experiment to area of control
		<i>per cent</i>	<i>days</i>			
6171 C	x-534	0.13	9	84	103	1.23
6172 C	x-534	0.13	9	42	61	1.45
6142 C	x-534	0.13	9	27	36	1.33
6140 C	x-534	0.13	9	24	53	2.20
6092 C	x-534	0.13	7	18	47	2.61
Average.....						1.76

TABLE VI.

Effect of the Addition of Gelatin Digest to Casein Digest on the Growth of Sarcomatous Fibroblasts of the Rat.

Experiment No.	Preparation No.	Nitrogen concentration	Time of growth	Area ⁷ of growth. Control ¹¹	Area ⁷ of growth. Experiment ¹²	Ratio: area of experiment to area of control
		<i>per cent</i>	<i>days</i>			
455 H	{ x-542 x-545	0.323	4	16	35	2.18
476 H	{ x-542 x-545	0.323	9	31	44	1.42
480 H	{ x-542 x-545	0.323	7	18	28	1.56
481 H	{ x-542 x-545	0.323	7	17	22	1.29
Average.....						1.61

⁹ The control medium consisted of 1 cc. of albumin digest and 2 cc. of Tyrode solution.

¹⁰ The experimental medium consisted of 1 cc. of albumin digest, 1 cc. of Tyrode solution, and 1 cc. of glycocoll solution.

¹¹ The control medium consisted of 1 cc. of casein digest and 1 cc. of Tyrode solution.

¹² The experimental medium consisted of 1 cc. of casein digest and 1 cc. of gelatin digest.

TABLE VII.

Effect of the Addition of Nucleic Acid to a Mixture of Crystalline Egg Albumin Digest⁸ and Glycocoll on the Rate of Growth of Sarcomatous Fibroblasts of the Rat.

Experiment No.	Preparation No.	Nitrogen concentration	Time of growth	Area ⁷ of growth. Control ¹³	Area ⁷ of growth. Experiment ¹⁴	Ratio: area of experiment to area of control	Remarks
		<i>per cent</i>	<i>days</i>				
6162 C	$\begin{cases} \text{x-534} \\ \text{x-551} \\ \text{x-554} \end{cases}$	0.1	9	58	108	1.87	1st passage
6162 C	$\begin{cases} \text{x-534} \\ \text{x-551} \\ \text{x-554} \end{cases}$	0.1	9	No growth	104		2nd "
6163 C	$\begin{cases} \text{x-534} \\ \text{x-551} \\ \text{x-554} \end{cases}$	0.1	9	53	110	2.07	
Average.....						1.97	

TABLE VIII.

Effect of the Addition of Nucleic Acid to a Mixture of Casein Digest and Glycocoll on the Rate of Growth of Sarcomatous Fibroblasts of the Rat.

Experiment No.	Preparation No.	Nitrogen concentration	Time of growth	Area ⁷ of growth. Control ¹⁵	Area ⁷ of growth. Experiment ¹⁶	Ratio: area of experiment to area of control
		<i>per cent</i>	<i>days</i>			
9488 D	$\begin{cases} \text{x-539} \\ \text{x-551} \\ \text{x-554} \end{cases}$	0.14	8	14	31	2.22
9492 D	$\begin{cases} \text{x-539} \\ \text{x-551} \\ \text{x-554} \end{cases}$	0.14	8	15	48	3.20
9441 D	$\begin{cases} \text{x-539} \\ \text{x-551} \\ \text{x-554} \end{cases}$	0.14	6	40	37	0.92
9454 D	$\begin{cases} \text{x-539} \\ \text{x-551} \\ \text{x-554} \end{cases}$	0.14	8	10	54	5.40
Average.....						2.93

¹³ The control medium consisted of 1 cc. of albumin digest, 1 cc. of glycocoll solution, and 1 cc. of Tyrode solution.

¹⁴ The experimental medium consisted of 1 cc. of albumin digest, 1 cc. of glycocoll solution, and 1 cc. of nucleic acid solution.

¹⁵ The control medium consisted of 1 cc. of casein digest, 1 cc. of glycocoll solution, and 1 cc. of Tyrode solution.

¹⁶ The experimental medium consisted of 1 cc. of casein digest, 1 cc. of glycocoll solution, and 1 cc. of nucleic acid solution.

TABLE IX.

Effect of the Addition of Casein Digest to a Mixture of Nucleic Acid and Glycocoll on the Rate of Growth of Sarcomatous Fibroblasts of the Rat.

Experiment No.	Preparation No.	Nitrogen concentration	Time of growth	Area ¹⁷ of growth. Control ¹⁷	Area ¹⁷ of growth. Experiment ¹⁸	Ratio: area of experiment to area of control
		<i>per cent</i>	<i>days</i>			
6196 C	$\begin{cases} \text{x-539} \\ \text{x-551} \\ \text{x-554} \end{cases}$	0.14	9	34	74	2.18
6219 C	$\begin{cases} \text{x-539} \\ \text{x-551} \\ \text{x-554} \end{cases}$	0.14	9	46	78	1.70
Average.....						1.94

TABLE X.

Effect of the Addition of Glycocoll to a Mixture of Casein Digest and Nucleic Acid on the Rate of Growth of Sarcomatous Fibroblasts of the Rat.

Experiment No.	Preparation No.	Nitrogen concentration	Time of growth	Area ¹⁷ of growth. Control ¹⁹	Area ¹⁷ of growth. Experiment ²⁰	Ratio: area of experiment to area of control
		<i>per cent</i>	<i>days</i>			
9463 D	$\begin{cases} \text{x-539} \\ \text{x-551} \\ \text{x-554} \end{cases}$	0.14	9	29	44	1.52
9472 D	$\begin{cases} \text{x-539} \\ \text{x-551} \\ \text{x-554} \end{cases}$	0.14	7	28	39	1.39
9443 D	$\begin{cases} \text{x-539} \\ \text{x-551} \\ \text{x-554} \end{cases}$	0.14	8	41	73	1.78
Average.....						1.56

¹⁷ The control medium consisted of 1 cc. of nucleic acid solution, 1 cc. of glycocoll solution, and 1 cc. of Tyrode solution.

¹⁸ The experimental medium consisted of 1 cc. of nucleic acid solution, 1 cc. of glycocoll solution, and 1 cc. of casein digest.

¹⁹ The control medium consisted of 1 cc. of casein digest, 1 cc. of nucleic acid solution, and 1 cc. of Tyrode solution.

²⁰ The experimental medium consisted of 1 cc. of casein digest, 1 cc. of nucleic acid solution, and 1 cc. of glycocoll solution.

TABLE XI.

Effect of a Mixture of Protein Digest, Glycocoll, and Nucleic Acid on the Growth of Normal Fibroblasts. Tyrode Solution Used as Control.

Experiment No.	Preparation No.	Nitrogen concentration	Time of growth	Area of growth. Control	Area of growth. Experiment	Ratio: area of experiment to area of control	Remarks
		<i>per cent</i>	<i>days</i>				
3847 A	{ x-539 x-551 x-554	0.14	5	66.5	95.4	1.43	Pure strain of chicken fibroblasts; casein digest
3830 A	{ x-534 x-551 x-554	0.10	6	28	56	2.00	Pure strain of chicken fibroblasts; crystalline egg albumin digest
3917 A	{ x-551 x-608 x-653	0.06	7	40	98	2.45	Pure strain of rat fibroblasts; crystalline egg albumin digest
3881 A	{ x-539 x-551 x-554	0.14	5	28	42	1.50	Pure strain of chicken fibroblasts; casein digest
9504 D	{ x-539 x-551 x-554	0.14	10	64	280	4.38	Pure strain of chicken fibroblasts; casein digest
144 G	{ x-551 x-554 x-562	0.14	6	14	42	3.00	Pure strain of chicken fibroblasts; casein digest
6298 C	{ x-539 x-551 x-554	0.14	8	70	135	1.93	Fibroblasts from chicken embryo heart; casein digest
6298 C	{ x-539 x-551 x-554	0.14	8	105	205	1.95	Fibroblasts from chicken embryo heart; casein digest
Average.....						2.33	

interesting to note that the digestion products of a vegetable protein, as well as those of animal origin, can be used by these cells. This was also found to be true of the gluten of wheat. In addition, normal chicken fibroblasts also proliferate in digests of both of these proteins.

Effects of Artificial Media on Normal Tissues.

Although the effect of adding glycocoll and nucleic acid to digests of proteins was tested on a strain of sarcomatous fibroblasts of the rat, it was also demonstrated that a pure strain of normal chicken fibroblasts will grow for a limited period in a mixture of protein digest, nucleic acid, and glycocoll. Text-fig. 7 gives the curve of growth for a strain of normal fibroblasts in casein digest, nucleic acid, and glycocoll. Similar results have been obtained with crystalline egg albumin digest, glycocoll, and nucleic acid. Although the rate of growth of normal fibroblasts is much larger in this artificial medium than in Tyrode solution (Table XI), the cells accumulate dark granulations in their cytoplasm, and die after 8 or 10 days. Liquefaction of the plasma clot often occurs. These cells seem to be more sensitive to the deficiencies of the artificial media than do the sarcomatous fibroblasts of the rat.

CONCLUSIONS.

1. The pepsin hydrolytic products of the pure proteins, crystalline egg albumin, crystalline edestin, and purified fibrin, are utilized by fibroblasts for their proliferation. It appears, therefore, that the growth of fibroblasts in fibrin digests and in the proteose of Witte's peptone is due to the split products of the protein itself and not to accompanying cellular constituents or other impurities. Preliminary denaturation of the protein, as carried out in these experiments, does not alter the nutritive properties of the digest.

2. The digests of the pure proteins employed are deficient in certain substances and do not meet the entire nutritive requirement of the cells for an unlimited period of time. Some supplementary nutritive substances are present in fresh embryonic heart tissue. This circumstance explains the growth of fibroblasts from fresh embryonic heart in digests which do not promote the growth of a pure strain of fibroblasts.

3. Glycocoll and nucleic acid have been found to supplement the nutritive action of pure protein digests for sarcomatous fibroblasts, and to increase greatly the length of life of the tissues.

4. Vegetable proteins, as well as animal proteins, yield proteolytic products which promote the multiplication of fibroblasts.

EFFECT OF LIVER AND PITUITARY DIGESTS ON THE PROLIFERATION OF SARCOMATOUS FIBROBLASTS OF THE RAT.

By LILLIAN E. BAKER, Ph.D., AND ALEXIS CARREL, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

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Since the experiments reported in the preceding paper¹ showed that the hydrolytic products of pure proteins do not support the indefinite proliferation of fibroblasts, and that they need to be supplemented by some substance present in fresh tissues, it seemed probable that a more complete nutritive medium might be obtained from the digests of certain tissues or glands instead of from pure proteins. The purpose of this paper is to describe the effects of the peptic digestion products of calf liver and the anterior lobe of the pituitary gland on the growth, not of fresh tissues, but of a pure strain of sarcomatous cells after several months of life *in vitro*.

Preparation of the Digests.

The liver digest was prepared by incubating 16 gm. of strictly fresh, finely ground liver for 20 hours at 37°C. in 200 cc. N/20 HCl, containing 1 gm. of Fairchild's pepsin and a few drops of toluene. After incubation, the pH was brought to 7.0 by the addition of N/1 NaOH and the solution boiled to half its volume. It was then rendered isotonic and adjusted to pH 7.4. The ratio of total to amino nitrogen in the completed preparation was 2.8. It contained 0.32 per cent nitrogen; of this nitrogen, 36.2 per cent was present as proteose, 56 per cent as peptone, and 7.8 per cent as lower split products.² There was no meta-protein present. About 7.8 per cent of the nitrogen was precipitated by 2 per cent trichloroacetic acid. This fraction has been classed as proteose³ rather than as protein, although

¹ Carrel, A., and Baker, L. E., *J. Exp. Med.*, 1928, xlvii, 353.

² Duplicate preparations vary to a slight extent. All of them, except one which contained considerable fatty material and a suspension of heat coagulated material too fine to centrifuge out, have supported a large growth of sarcomatous fibroblasts.

³ It is believed that the substances precipitated by 2 per cent of trichloroacetic acid cannot be classed as protein, but rather as proteose, for the authors obtain

Wasteneys and Boorsook, whose method of analysis was employed, are of the opinion that it should be classed as protein.⁴ Before being used in the culture medium, this digest was diluted with 7 parts Tyrode solution, making the concentration of nitrogen 0.04 per cent.

The pituitary digest⁵ was prepared by incubating 12 gm. of the finely ground anterior lobe of pituitary glands of calves or steers in 120 cc. of 0.5 per cent pepsin in N/20 HCl for 16 hours. After digestion, the pH was brought to 7.0 and the solution boiled to half its volume, centrifuged, rendered isotonic, and finally adjusted to pH 7.4. The completed preparation contained 0.37 per cent total nitrogen, 36.3 per cent of which was present as proteose. The ratio of total to amino nitrogen was 3.4. The mixture was diluted with Tyrode solution to varying concentrations (Table I) before being used in the culture medium.

Preparation of Cultures.

The digests were tested on a pure strain of malignant fibroblasts of the rat, isolated from Crocker Foundation Sarcoma 10. The colonies were cut into two equal parts, one of which was cultivated in embryo juice as a control, and the other in the experimental fluid. Flasks 3 cm. in diameter were used. The plasma clot was formed by mixing 1 cc. of plasma previously diluted with 2 parts of Tyrode solution and a few drops of embryo juice. As soon as coagulation took place, the clot was washed for half an hour with 3 cc. of Tyrode solution and then 0.5 cc. of the experimental and control fluids was added. Every 48 hours, the cultures were washed with 3 cc. of Tyrode solution for half an hour, 0.5 cc. of nutritive fluids was added, and drawings were made, under a projectoscope, of the area of growth.

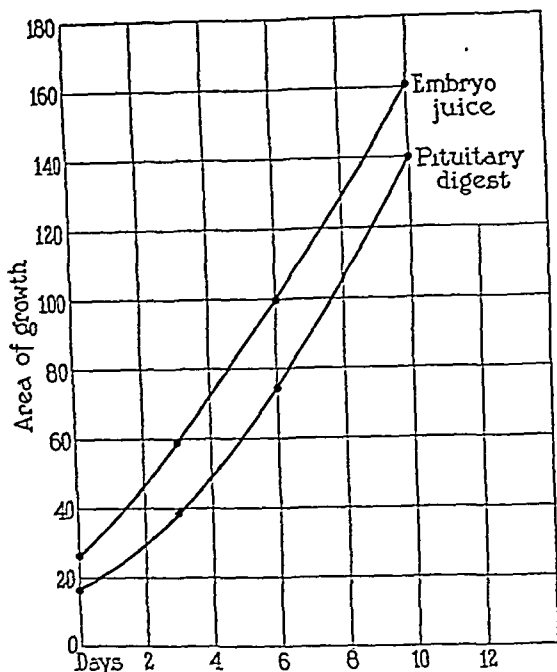
Action of the Pituitary Digest on Sarcomatous Fibroblasts.

The rates of growth of sarcomatous fibroblasts in the peptic digests of the anterior lobe of the pituitary and of their controls in embryo juice were approximately equal. Text-fig. 1 gives typical growth curves for the first passage of these tissues in a digest of calf pituitary

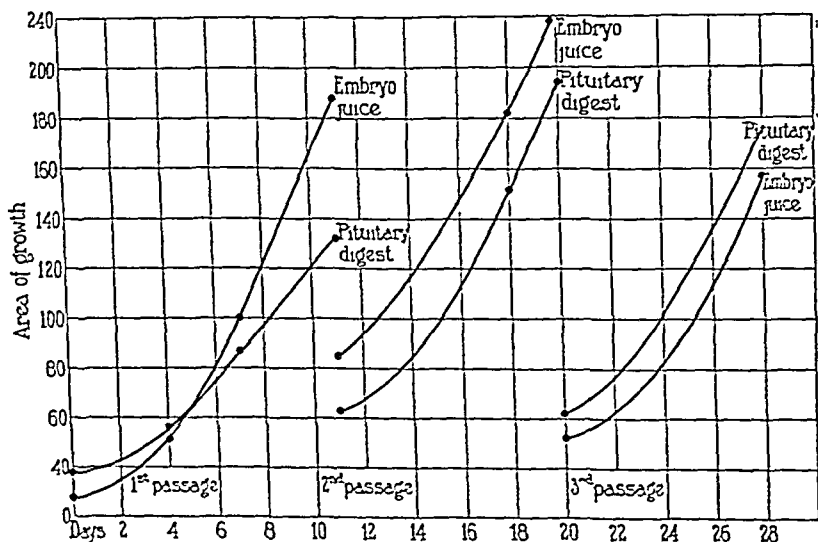
a bulky precipitate containing 10 per cent of the total nitrogen present in digests of crystalline egg albumin which have been boiled previously to remove any remaining albumin. It was also shown that this precipitate was not meta-protein, as the amount of meta-protein in the solutions was so small as to be almost negligible. Obviously, therefore, trichloroacetic acid precipitates proteolytic products other than protein and meta-protein. This is in accord with the work of Hiller, A., and Van Slyke, D. D., *J. Biol. Chem.*, 1922, liii, 253.

⁴ Wasteneys, H., and Boorsook, H., *J. Biol. Chem.*, 1924, lxii, 1.

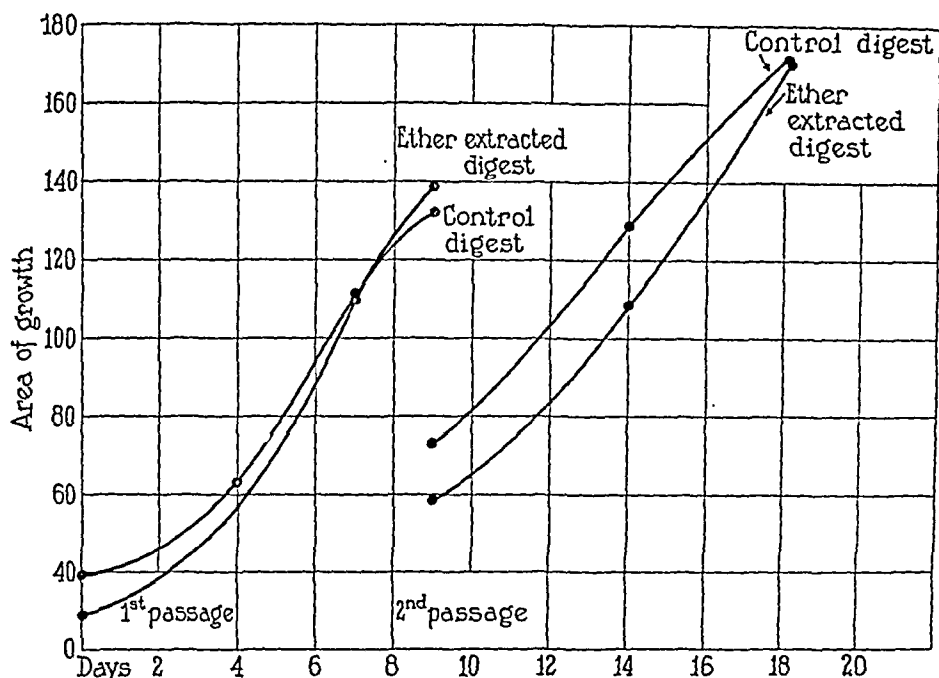
⁵ Duplicate preparations of these digests have varied slightly in composition. All have, however, caused a large growth of sarcomatous fibroblasts.



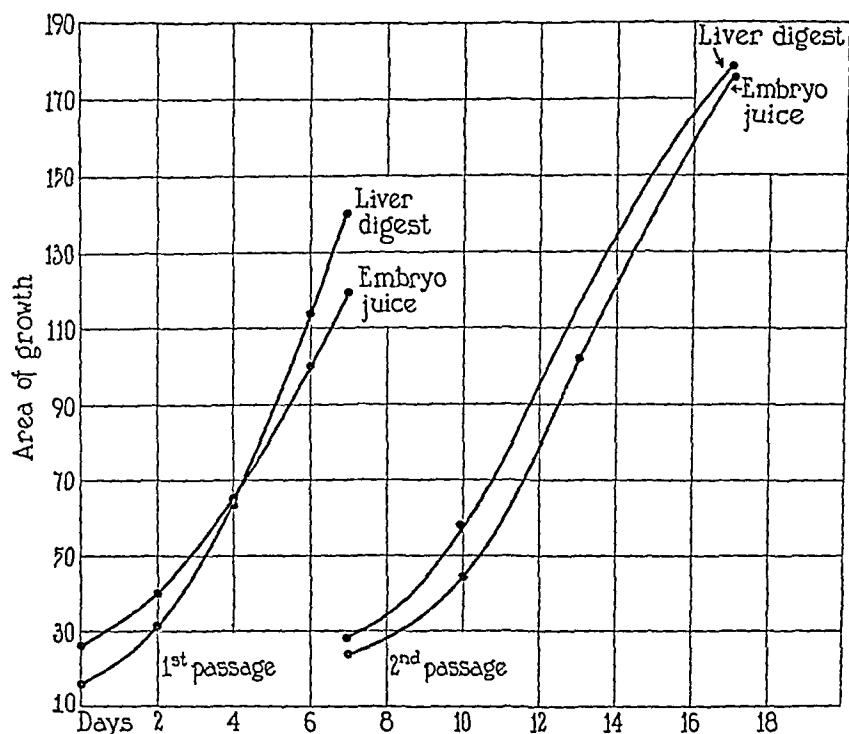
TEXT-FIG. 1. Experiment 280 H. Rate of growth of sarcomatous fibroblasts of the rat in a digest of the anterior lobe of pituitary gland, and in embryo juice.



TEXT-FIG. 2. Experiment 6211 C. Rate of growth of sarcomatous fibroblasts of the rat during three successive passages in an ether extracted digest of anterior lobe of pituitary gland. Control tissues in embryo juice.



TEXT-FIG. 3. Experiment 6130 C. Rate of growth of sarcomatous fibroblasts of the rat in a digest of the anterior lobe of the pituitary and in the same digest extracted ten times with ether.



TEXT-FIG. 4. Experiments 9546 D and 9590 D. Rate of growth of sarcoma-fibroblasts of the rat during two passages in calf liver digest.

and in embryo juice. The results of sixteen experiments at different concentrations of nitrogen are summarized in Table I. Similar results were obtained with many different preparations. No differences were observed between the action of the digests of calf pituitary and of those of steer. Not only was the rate of growth in the pituitary digest as great as in embryo juice, but the cells remained in excellent

TABLE I.

Comparison of the Rates of Growth of Sarcomatous Fibroblasts of the Rat in Embryo Juice and in a Digest of Anterior Lobe of Pituitary.

Experiment No.	Preparation No	Nitrogen concentration	Time of growth	Area* of growth in embryo juice. Control ¹	Area* of growth in pituitary digest. Experiment	Ratio of area of growth in experiment to area in control	Remarks
		<i>per cent</i>	<i>days</i>				
280 H	x-496	0 25	10	135	124	0 91	Steer pituitary
232 H	x-496	0 25	8	63	65	1 03	" "
272 H	x-496	0 25	8	81	94	1.16	" "
4979 C	x-518	0 016	9	85	64	0 75	Calf "
4979 C	x-518	0 016	9	67	66	0 99	" "
292 H	x-518	0 032	7	82	72	0 88	" "
292 H	x-518	0 016	7	42	68	1 62	" "
4923 C	x-518	0 06	10	90	85	0 94	" "
298 H	x-518	0 20	11	127	114	0 90	" "
293 H	x-519	0 03	7	38	36	0 95	Steer "
298 H	x-519	0 20	11	115	114	0 99	" "
6143 C	x-519	0 08	8	90	88	0 98	" "
4923 C	x-519	0 25	10	66	64	0 97	" "
4923 C	x-519	0.06	10	29	52	1 79	" "
320 H	x-519	0 03	12	78	65	0 83	" "
5031 C	x-513	0 03	9	61	62	1 02	Calf "
Average ratio						1 04	

condition as far as could be observed, and did not show the fatty degeneration characteristic of cells cultivated in pure protein digests. The same colonies continued to proliferate through many successive passages at a uniform rate. Text-fig. 2 shows three successive passages of a colony in a digest that had been thoroughly extracted with

¹ This is the area in square centimeters of the projected image of the colony of cells, and is 62 times the size of the colony.

ether. It was thought that possibly the lipoids of the glands were furnishing some substance required by the cells which might explain, in part at least, the difference between digests of pure proteins and those of glandular material,⁷ but evidently this is not the case. The cells multiplied as rapidly in digests of the pituitary which had been extracted ten times with ether as in embryo juice (Table II), or in a part of the same digest which was not extracted (Text-fig. 3, Table III).

TABLE II.

Rates of Growth of Sarcomatous Fibroblasts of the Rat in Ether Extracted Digest of Anterior Lobe of Pituitary and in Embryo Juice.

Experiment No.	Preparation No.	Nitrogen concentration	Time of growth	Area ^s of growth in embryo juice. Control	Area ^s of growth in pituitary digest. Experiment	Ratio of area of growth in experiment to area in control	Remarks
		<i>per cent</i>	<i>days</i>				
6132 C	x-485	0.03	8	118	92	0.78	1st passage
6158 C	x-485	0.03	10	104	107	1.03	" "
6115 C	x-484	0.03	11	86	86	1.00	" "
6115 C	x-484	0.03	9	No control	93		2nd "
6115 C	x-484	0.03	8	" "	96		3rd "
6115 C	x-484	0.03	6	" "	57		4th "
6211 C	x-484	0.06	11	160	100	0.63	1st "
6211 C	x-484	0.06	10	145	130	0.90	2nd "
6211 C	x-484	0.06	9	127	114	0.90	3rd "
Average ratio.....						0.87	

Action of the Liver Digests on Sarcomatous Fibroblasts.

The digests of liver gave even better results. In many cases, the rate of multiplication of the sarcomatous fibroblasts in the liver digest exceeded that of their controls in embryo juice (Table IV). The growth remained uniform throughout many successive passages (Text-fig. 4), with no change that could be noticed in the condition of the cells. A strain of these fibroblasts was kept in this medium for over 3 months, *i.e.*, through ten passages, and was still proliferating as actively as those which had been cultivated in embryo juice.

⁷ The experiments reported in the preceding paper¹ indicate that the presence of nucleic acid in digests of glandular material accounts in part for this difference.

TABLE III.

Rates of Growth of Sarcomatous Fibroblasts of the Rat in a Digest of the Anterior Lobe of Pituitary and in the Same Digest Extracted with Ether Ten Times.

Experiment No.	Preparation No.	Nitrogen concentration	Time of growth	Area ^a of growth in pituitary digest. Control	Area ^a of growth in ether extracted digest. Experiment	Ratio of area of growth in experiment to area in control	Remarks
		<i>per cent</i>	<i>days</i>				
6130 C	x-556	0.04	9	104	99	0.95	1st passage
6130 C	x-556	0.04	9	99	115	1.16	2nd "
6133 C	x-556	0.02	9	77	95	1.23	1st "
9424 D	x-556	0.02	6	29	28	0.96	" "
9444 D	x-556	0.04	8	56	50	0.89	" "
Average ratio.....						1.04	

TABLE IV.

Rate of Growth of Sarcomatous Fibroblasts of the Rat in Calf Liver Digest. Controls Cultivated in Embryo Juice.

Experiment No.	Preparation No.	Nitrogen concentration	Time of growth	Area ^a of growth in embryo juice. Control	Area ^a of growth in liver digest. Experiment	Ratio of area of growth in experiment to area in control	Remarks
		<i>per cent</i>	<i>days</i>				
9513 D	x-583	0.04	17	180	162	0.90	1st passage
9513 D	x-583	0.04	9	170	100	0.59	" "
9623 D	x-583	0.04	7	38	80	2.11	2nd "
9667 D	x-583	0.04	5	100	50	0.50	3rd "
9530 D	x-583	0.08	12	118	144	1.22	1st "
9622 D	x-583	0.08	7	57	79	1.39	2nd "
9666 D	x-583	0.08	5	96	113	1.18	3rd "
9546 D	x-583	0.08	7	92	129	1.40	1st "
9590 D	x-583	0.08	10	157	153	0.97	2nd "
9590 D	x-583	0.08	10	126	151	1.20	" "
9631 D	x-583	0.08	6	51	87	1.71	3rd "
9631 D	x-583	0.08	6	48	100	2.08	" "
9659 D	x-583	0.08	6	37	109	2.95	4th "
Average ratio.....						1.40	

Action of Liver and Pituitary Digests on Normal Fibroblasts.

When pure strains of normal chicken and normal rat fibroblasts were cultivated in the liver and pituitary digests, considerable proliferation of cells occurred. The rate of growth was not as great as in embryo juice and the cells finally underwent fatty degeneration. The normal tissue appears to be more sensitive to the deficiencies of the medium than the sarcoma tissue. Further experimentation is necessary before conclusions can be drawn concerning this difference in normal and sarcomatous cells.

SUMMARY.

1. A media containing all the essential constituents for the cultivation *in vitro* of sarcomatous fibroblasts of the rat has been prepared by digesting calf liver and also the anterior lobe of the pituitary body with pepsin.

2. The nutritive action of the pituitary digests is not altered by thorough extraction with ether.

3. After a pure strain of sarcomatous fibroblasts had been cultivated for 3 months in a liver digest, its proliferative activity was as great as at the beginning of the experiment. The same was true of the colonies cultivated for 1 month in a digest of pituitary gland. The increase in the volume of the colonies which takes place in the digests is about as great as that produced by chick embryo juice.

4. Normal chicken fibroblasts also proliferate in both digests, but they undergo fatty degeneration after a more or less prolonged period of cultivation.

ON THE RÔLE OF CARBOHYDRATE HAPTENS IN BACTERIAL ANAPHYLAXIS.

By JOSEPH TOMCSIK, M.D., AND T. J. KUROTCHKIN, M.D.

(From the Department of Pathology, Peking Union Medical College, Peking, China.)

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Though the existence of bacterial anaphylaxis was demonstrated by Rosenau and Anderson (1) as early as 1907, subsequent investigators could not diminish the difficulties in producing a clear-cut anaphylactic shock with the majority of microorganisms. The main difficulty in the study of bacterial anaphylaxis is the primary toxicity of the bacterial protein. According to Doerr (2) the amount of bacterial antigen which produces shock in sensitized animals is negligibly smaller than the lethal dose for normal animals. More recently Zinsser and Parker (3) found that in guinea pigs sensitized passively with typhoid immune serum at least a minimal fatal dose of the typhoid extract is required to elicit in sensitized animals acute shock with death.

In the study of bacterial anaphylaxis many investigators have used suspensions of organisms, not extract, so the *in vivo* tests are complicated by the fact that these suspensions may produce symptoms even in untreated animals. This complication can be obviated by using the Schultz-Dale method. Sherwood and Stoland (4) working with typhoid bacilli found that in some cases the sensitized uterus contracted after the addition of 0.25 cc. of bacterial extract, whereas the minimum toxic dose was 1 cc. Since the uteri of the sensitized animals failed to react in many cases, they conclude, on the basis of comparative examinations, that more positive results can be obtained with the *in vivo* method and that the Dale test is not a necessary criterion of sensitization. Zinsser and Mallory (5) do not share this unfavorable opinion. They find that uterine reactions, like those obtained after sensitization with proteins, can be demonstrated in guinea pigs after both active and passive sensitization against alkaline extracts of pneumococci, but "The margin between the minimum doses which contract the normal and those which contract the sensitized uteri is incomparably smaller than that obtained in analogous experiments with . . . egg albumen." With regard to the difficulty of demonstrating bacterial anaphylaxis Zinsser and Mallory state: "it would seem to us that active sensitization is so difficult because the bacteria contain relatively little coagulable protein, and that in passive sensitization there is an apparently slower union of antibodies with the animal tissues than in the case of protein antibodies." If this assumption is right we have little hope of improving our methods for the study of bacterial anaphylaxis, the difficulties being inherent in the nature of this phenomenon. It is conceivable however, that the primary toxic substance

of the bacteria is not necessarily identical with the specific antigenic part and that by suitable chemical separation it may be possible to isolate an atoxic specific constituent, responsible for the production of anaphylactic shock. With the discovery of specifically reacting polysaccharides in certain microorganisms a new field was opened for investigation. These substances, immunologically haptens, give powerful precipitation and complement fixation reactions with immune serum, but they do not lead to the formation of antibodies, when injected into animals. According to Doerr the anaphylactic antibodies against bacterial proteins are probably identical with the precipitins, since the power of an immune serum to confer passive sensitization is in proportion to its precipitin content. If this is true we should be able to produce anaphylactic shock with a carbohydrate hapten, since it has a remarkably high activity in precipitin reactions.

As far as we are aware no experiments have been reported which show a difference between the sensitizing and shock-producing part of the antigen in typical anaphylaxis. However in the classical investigations of Zinsser and his associates it appears that such a difference exists in the related field of bacterial allergy. Zinsser and Parker (6) produced typical skin reactions with residue antigen in guinea pigs infected with tuberculosis. In later work Zinsser and Mueller (7), then Zinsser and Tamiya (8) succeeded in isolating from *Bacillus tuberculosis* and the pneumobacillus an active material which produced a positive skin test and yet appeared to be different from the soluble specific substance. Both of these substances lack true antigenic activity, the substance producing skin reactions being a protein-free nitrogenous material, while the soluble specific substance is a carbohydrate. This work was not extended to the study of typical anaphylaxis, probably because it was a generally accepted opinion that true protein anaphylaxis plays an unimportant rôle in the occurrence of spontaneous infections.

The object of the present work was to determine whether animals could be sensitized to carbohydrate haptens, either actively by the injection of bacteria, or passively by immune serum. Sensitization has been determined by the injection of the hapten intravenously and by the reaction of the uterus according to the Schultz-Dale technic.

Bacillus lactis aerogenes was chosen for the major part of our study. The isolation of the specific substance from this bacterium and its chemical nature has been described by one of us previously (9). It consists chiefly of carbohydrates containing, after hydrolysis, 66 per cent reducing substance, counted as glucose. It is protein-free, but in spite of several attempts at further purification, its nitrogen content could not be reduced below 0.9 per cent. It gave specific precipitation reaction, when diluted in 1:500,000 and complement fixation reaction when diluted as high as 1:64,000,000.

EXPERIMENTAL.

In Vivo Tests in Actively Sensitized Animals.

Attempts were made at the beginning of our work to produce active sensitization against the *aerogenes* specific substance.

Holobut's method (10) was adopted in sensitizing the animals, using massive intraperitoneal inoculations of living or killed organisms on 10 consecutive days and testing the sensitivity 3 weeks after the last injection. The difficulty we encountered was the high toxicity of the bacillus for guinea pigs. In the first lot of 12 guinea pigs, 6 were inoculated with living bacteria and 6 with bacteria killed at 60°C. Each animal died after the second or the third injection, when the single dose was 1/20th of the growth from a 24 hour agar culture. In inoculating the second lot of guinea pigs, smaller doses were used, beginning with 1/200th and slowly increasing to 1/60th part of a 24 hour agar culture. 6 guinea pigs were inoculated with living and 6 with killed bacteria and daily injections were given on 9 consecutive days. At the end of this treatment 3 animals survived from each group. These were tested for sensitivity 3 weeks after the last injection, in part by intravenous inoculation of 1 mg. *aerogenes* specific substance, in part by testing the isolated uterus. No reaction was observed in any of these tests.

Passive Sensitization toward the Aerogenes Specific Substance.

Our experiments in regard to active sensitization were not extensive enough for the formulation of any conclusion. In view of the difficulty of obtaining sensitization in this way we limited ourselves in further work to the study of passive anaphylaxis. One part of these experiments was reported by one of us in a preliminary paper (11).

The immune serum we have used for the passive sensitization was prepared in rabbits by six to eight intravenous injections of the killed suspension of *Bacillus lactis aerogenes*. The interval between the injections was either 3 or 4 days. The rabbits were bled 8 days after the last injection.

In performing the sensitization, guinea pigs weighing 250 to 380 gm. were inoculated intraperitoneally with from 1 to 4 cc. immune serum. After 16 to 24 hours, 1 cc. of the various dilutions of the specific substance in saline was injected in the saphenous vein. Each of the 18 sensitized guinea pigs tested intravenously with 1 to 0.033 mg. of the specific substance died, showing typical anaphylactic symptoms. Death usually occurred within 2 to 3 minutes after the injection and never later than 5 minutes. The lungs were markedly distended and pale. 1 guinea pig receiving 0.02 mg. specific substance showed symptoms but survived.

Control tests were made on 11 untreated guinea pigs weighing 250 to 300 gm. None of these animals showed any immediate symptoms. When the dose was as

high as 1 to 2 mg. some of the animals died on the 2nd or 3rd day, without showing any symptoms other than drowsiness and emaciation. At the autopsy no changes were observed. Smaller amounts than 1 mg. never caused any symptoms in untreated animals. The slight toxicity of the aerogenes specific substance in normal animals can be explained probably by the presence of a small amount of nitrogenous material. The margin between the primary toxic dose (2 mg.) and the amount which produced a fatal shock (0.033 mg.) was much wider than that found previously with bacterial extracts.

It should be noted that there was not a single experiment in which we failed to produce a fatal shock in sensitized animals, provided the amount of the extract inoculated intravenously was not less than 0.033 mg.

In tests to confirm the results obtained *in vivo*, the smooth muscle reaction was used in further work. 20 guinea pigs weighing 210 to 260 gm. were sensitized by the intraperitoneal injection of 1 cc. aerogenes immune serum and the responses of their uteri tested, using the Schultz-Dale method. The uteri of all these animals reacted to the specific substance, when tested 2 hours to 12 days after the injection of serum. According to earlier authors the optimal incubation time for the development of passive anaphylaxis is about 1 day. Zinsser and Parker (3) in their experiments with typhoid bacilli never succeeded in finding the animals sensitized in less than 3 to 5 days, the highest degree of sensitization being developed in 1 week. Using the aerogenes specific substance we did not observe appreciable difference in the sensitivity even when the incubation time varied between 2 hours and 12 days. In establishing accurately the smallest amount of specific substance which causes a contraction in the sensitized uterus, one difficulty was observed, apart from the individual variations of the uteri. The two horns of a uterus were suspended approximately at the same time in the two baths. To the first horn 0.5 cc. of a 1:80,000 dilution of the specific substance was added, without causing any contraction, but 1:50,000 dilution caused a distinct reaction of the other horn. After changing the bath 1:80,000 dilution added to the second horn caused a reaction with a curve similar to that following the previous larger dose. That is a slighter preliminary reaction increased the sensitivity of the uterus. This observation has been previously made by Weil (12) in his studies in protein anaphylaxis.

The smallest amount of the specific substance which caused a distinct, but not maximal contraction, was 0.00625 mg. added to a bath of 125 cc. The final concentration of the substance therefore was 1:20,000,000.

Desensitization of the uterus was demonstrated after the contraction due to one addition of 5 mg. of the substance to the bath. If, however, 1 mg. of the specific substance was used, three subsequent doses were required, when the bath was changed after each contraction, to obtain a complete desensitization.

As controls the uteri from 6 normal guinea pigs were tested and not one gave a reaction following the addition of as much as 10 mg. of the specific substance to the bath. When tested for sensitivity to specific substances obtained from other types of aerogenes, there was no response indicating overlapping specificity so that the results correspond to those obtained with the precipitin reaction.

These experiments seem to justify the conclusion that passive anaphylaxis toward a non-protein bacterial extract can be demonstrated with the same degree of sensitiveness and specificity as observed in protein anaphylaxis.

It might be mentioned briefly that in a few cases the skin test was tried in passively sensitized animals, though these experiments do not warrant any definite conclusion.

11 guinea pigs were sensitized by the intraperitoneal injection of 1 to 5 cc. immune serum. After 24 hours 0.1 cc. of 1:500 or 1:1000 dilution of the specific substance was injected into the abdominal skin. In most of these animals a moderate swelling developed in 3 to 5 hours, the largest being 2 cm. in diameter, with an inflammatory red area in the middle. The reaction disappeared in 12 to 18 hours. In 7 unsensitized animals no changes were observed in the injected area.

The type of skin reaction we observed in the sensitized animals can be classified neither as an immediate, evanescent urticarial reaction, characteristic of protein anaphylaxis, nor as a late allergic reaction, since it disappears in 24 hours. We feel that additional work is needed to establish the nature of this reaction.

Passive Sensitization to the Carbohydrate Hapten of the Pneumobacillus.

In this further work, our object was to confirm the results obtained with the aerogenes specific substance, using the carbohydrate haptens

of other microorganisms which can be purified more highly. Accordingly the specific substance was isolated from a strain of pneumobacillus, originally obtained from a normal throat.

This hapten was isolated first by Heidelberger, Goebel, and Avery (13) and after a long and careful process of purification, it was found by them to be free from nitrogen and to consist of a polysaccharide built up from glucose units. For technical reasons we could not follow the method used by these authors, but adopted Toeniesen's method (14). The raw extract thus obtained, which corresponds probably to the capsular material, was subjected to subsequent purification. At the beginning of our work the capsule of the freshly isolated strain was exceptionally abundant; 3 or 4 months later, after repeated transfer on meat infusion agar medium, it became much less. At this stage the yield of the extract was also smaller, but did not differ in reactivity from the first extract. It gave precipitation with a pneumobacillus rabbit immune serum, when diluted in 1:300,000. From the bacterial growth of 100 Kolle flasks 1.2 gm. of extract was obtained, in the form of a white powder easily soluble in water. A weakly alkaline solution of this substance in a dilution of 1:200 was precipitated 6 times in succession with 3 volumes of absolute alcohol. The resulting powder gave a specific precipitin reaction, when diluted 1:500,000 and layered over immune serum. Additional purification was carried out using the uranyl nitrate precipitation method. In this we followed very closely the technic given by Heidelberger, Goebel, and Avery (13). The resulting powder was dissolved in water and dialyzed for 2 days in a parchment bag, then filtered through Seitz filter, and precipitated by alcohol. In the final stage it was precipitated by the corresponding immune serum when diluted in 1:2,000,000. It gave no precipitation with any of the pneumococcus type sera. In the presence of 1:20 dilution of the immune serum it gave complement fixation when diluted 1:32,000,000.

After hydrolysis it contained 71.5 per cent reducing substance counted as glucose. When the hydrolysis was made in normal sulfuric acid solution, the optimal time was found to be 9 hours. The nitrogen content of this specific substance was 0.26 per cent, considerably lower than that of the aerogenes extract.

The method for demonstrating anaphylaxis to the pneumobacillus carbohydrate was the same as that described above with the aerogenes specific substance.

Guinea pigs were sensitized by the intraperitoneal injection of 1 cc. pneumobacillus immune serum and 24 hours later injected intravenously with various dilutions of the specific substance. The smallest amount of the carbohydrate extract which produced invariably a typical anaphylactic shock with death was found to be 0.01 mg. 5 untreated guinea pigs were used as controls and failed to show any symptoms after the injection of amounts as large as 2 mg.

These results seem to us, to be especially convincing since the carbohydrate extract did not possess any primary toxicity and yet it produced anaphylactic shock even when given in smaller amounts than had been used previously with proteins. According to Wells (15) the smallest amount of protein capable of causing anaphylactic shock is 0.1 to 0.05 mg.

The results *in vivo* were confirmed by the Schultz-Dale reaction. A 1:12,500,000 dilution of the carbohydrate in the bath was enough to produce contractions in sensitized uteri. It is interesting that a single contraction due to the addition of 1 mg. carbohydrate to the bath was enough to desensitize the muscle completely.

Experiments with Yeast Gum.

Studies in yeast anaphylaxis have been made by Rosenau and Anderson (1) and by Axamit (16). Yeast cells or crude extracts were used in testing the sensitivity of the treated animals. The slight difference between the primary toxic and the shock-producing doses made the interpretation of these results just as difficult as in the case of bacterial anaphylaxis.

Our object was to test passively sensitized guinea pigs with yeast gum, the hapten nature of which has been shown by Mueller and by one of us (17).

The strain of yeast used in this work was obtained from a case of stomatitis. The yeast cells grown on Sabouraud culture medium were collected and freed from the constituents of the culture medium by alcoholic precipitation. The extraction was made by the hot alkaline method. From this extract the gum was obtained by the usual procedure, precipitating with Fehling reagent. The last trace of the copper was taken out by repeated alcoholic precipitation and dialysis.

In the final stage of purity 83 per cent of the gum consisted of reducing substances counted as glucose. Nitrogen was present in trace, approximately 0.2 per cent.

Passive anaphylaxis toward the yeast gum was studied both *in vivo* and with the uterine reaction. Since the results of these experiments entirely correspond to those obtained with the specific substances of *Bacillus lactis aerogenes* and of the pneumobacillus, their description can be omitted to avoid repetition. The only difference observed was that repeated additions of larger amounts of yeast gum were

necessary to produce desensitization of the uterus, whereas this was easily accomplished with pneumobacillus extract. The injection of the yeast gum into six untreated animals proved to be without effect.

TABLE I.

Summary of the Reactions Obtained with the Specific Substances of B. lactis aerogenes, Pneumobacillus, and Yeast.

		Specific substances from		
		<i>B. lactis aerogenes</i>	Pneumobacillus	Yeast
Nitrogen.....		0.9 per cent	0.26 per cent	0.2 per cent
Reducing substances after hydrolysis, counted as glucose.....		66 per cent	71.5 per cent	83 per cent
Precipitin titer.....		1:500,000	1:2,000,000	1:1,000,000
Titer in complement* fixation.....		1:64,000,000	1:32,000,000	1:32,000,000
<i>In vivo</i> tests	Toxic dose for normal guinea pigs.....	1 mg.	2 mg. non-toxic	2 mg. non-toxic
	Minimal shock-producing dose.....	0.033 mg.	0.01 mg.	0.02 mg.
<i>In vitro</i> tests with Dale technic	Largest amount failing to cause contraction of normal uterus.....	10 mg.	10 mg.	10 mg.
	Smallest amount causing contraction of the uterus from sensitized guinea pigs.....	0.00625 mg.	0.01 mg.	0.025 mg.
	Amount causing desensitization.....	5 mg.	1 mg.	Not complete after 10 mg.

* Graded amounts of the antigens were tested in the presence of 0.5 cc. 1:20 dilution of immune serum and 2 units of complement. Incubation period was 16 hours at 6° to 8°C. The amount in the last tube which did not show a trace of hemolysis was regarded as the titer.

DISCUSSION.

The experiments described above show that small amounts of protein-free hapten obtained from *B. lactis aerogenes* will produce anaphylactic shock, both *in vivo* and *in vitro*, in guinea pigs which have been passively sensitized with immune serum from rabbits injected

with dead cultures of the same organism. In the same way, haptens from the pneumobacillus and from a yeast caused typical shock in animals sensitized by the appropriate immune sera. When haptens are used to bring out the reaction, the wide margin between the toxic and the minimal shock-producing doses corresponds to that found in protein anaphylaxis. Desensitization is produced in the isolated uterus by the addition of the hapten to the bath. The muscle is of course washed and rested before the desensitization test is made.

Repeated injections of living and killed cultures have not produced a sensitization to the hapten in the few animals that survived the treatment.

Just as haptens fail to cause the production of precipitins and complement-fixing bodies, so do they fail to sensitize guinea pigs when injected in their pure state. That they may play a part in sensitization when combined with protein seems possible.

The haptens used in these experiments gave none of the protein reactions, and while they were not absolutely nitrogen-free, this element was almost negligible in the pneumobacillus and yeast preparations. Since shock was produced by smaller amounts of hapten than will cause a reaction in animals sensitized to purified proteins, it does not seem possible that the small amount of nitrogen present played any part in the reaction. A positive statement is unjustified because of the trace of nitrogen present, but we believe that we have shown in these experiments that anaphylactic shock can be produced by carbohydrates.

Since the serum used for the sensitizations had high contents of precipitin and complement-fixing bodies which react with minute amounts of the appropriate hapten, we feel that the results obtained support the view that precipitins and complement-fixing bodies are closely related to the bodies which have to do with sensitization, if not identical with them.

CONCLUSIONS.

1. In passively sensitized animals bacterial anaphylaxis has been produced, *in vivo* and *in vitro*, with haptens from *B. lactis aerogenes*, the pneumobacillus, and a yeast.
2. The smallest amount of hapten causing fatal anaphylaxis is less

than the minimal amount of protein which will cause death in properly sensitized animals.

3. The haptens used were largely carbohydrates, and gave none of the protein reactions, but since they did contain a small amount of nitrogen we cannot yet assert positively that carbohydrate alone will produce shock.

4. Since haptens will not sensitize animals we must conclude that the anaphylactogenic and shock-producing parts of the antigen are not identical.

5. These experiments provide further evidence of the close relationship of precipitins to anaphylaxis.

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STUDIES ON THE INACTIVATION OF VACCINE VIRUS AND THE ACTION OF CERTAIN SUBSTANCES UPON THE INFECTING POWER OF THE INACTI- VATED VIRUS.*

By F. DURAN REYNALS, M.D.†

(From the Laboratories of The Rockefeller Institute for Medical Research.)

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The present status of the discussion opened by the experiments of Gye and Barnard (1, 2) supporting the virus theory of cancer, can be summarized as follows. While certain investigators have been able to repeat Gye's results and are in agreement with his interpretations (3, 4), others have failed (5-8). Murphy (9) and more recently Flu (10) and Cori (11) duplicated Gye's work but by means of more careful control experiments have added a new fact of basic importance, namely the non-specific reactivation of the chloroformed sarcoma filtrate (specific factor) by substances other than the cultures of malignant tissues. It is obvious that Gye's theory does not hold in the light of these later facts, although Gye himself claims to get only negative results in 150 control experiments of a similar nature.

In the opinion of Murphy also shared by Flu, the interpretation of these results is not that a virus has been rendered infective by a specific factor but that an unknown substance of tissue origin enables the agent, modified or attenuated by chloroform, to act. Flu suggests that these substances are similar to bacterial aggressins. Simon and Beck (6) explained Gye's results on the basis of an aggregation of subinfective doses of the agent itself or of a non-specific reactivation, while Harde (12) suggests that the activator is effective only through its acidity.

In order to throw further light on this subject we have carried out

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† Fellow of the Spanish Government (Junta para Ampliación de Estudios).

an investigation similar to that of Gye but have substituted a typical virus in the place of the etiological agent of the chicken sarcoma. Our plan has been to determine whether the vaccine virus attenuated to such an extent that it would produce no lesion could be rendered infective by the addition of certain substances.

Methods and Materials.

Vaccine Virus.—In all of the experiments the same strain of vaccine virus has been used.¹ Half a cc. of the vaccine emulsion mixed with an equal amount of Ringer's solution was injected into both testicles of a rabbit. Five days later when the resulting orchitis was at its height the animal was killed and the testicles removed

TABLE I.

Tube	Amount of chloroform	Resulting lesion	Tube	Amount of ether	Resulting lesion
	cc.			cc.	
1	0.2	—	4	0.2	++
2	0.15	—	5	0.15	++
3	0.1	+	6	0.1	++

TABLE II.

Tube	Amount of chloroform	Resulting lesion	Tube	Amount of ether	Resulting lesion
	cc.			cc.	
1	0.15	—	5	0.2	++
2	0.1	±	6	0.15	++
3	0.05	+	7	0.05	+++
4	0.01	+			

aseptically. They were ground thoroughly with sand together with 25 cc. of glycerol and Ringer's solution. The resulting emulsion was distributed in tubes, covered with a layer of sterile vaseline and kept in the ice box.

In order to test the activity of the virus the emulsion was diluted with Ringer's solution and 0.2 cc. injected intradermally in rabbits. With a 1 to 500 dilution of the emulsion (about 1 to 5000 of the testicular material) a slight but definite

¹ We wish to acknowledge our indebtedness to Dr. T. M. Rivers for supplying the original strain of vaccine virus and for his suggestions and criticisms during the course of this investigation.

vaccine eruption appeared in 4 or 5 days. The injection of 0.2 cc. of a 1 to 10 dilution gave rise in the same time to a greatly congested circular eruption 2.5 to 3 cm. in diameter. This latter dose was the one generally used in the experiments.

Inactivation of Virus.

The same general methods have been employed as were used in Gye's experiments. Not only chloroform but other organic solvents were tested as to their effect on the vaccine virus.

Method.—To 10 cc. of the vaccine emulsion diluted 1 to 10 and placed in a 50 cc. centrifuge tube, various amounts of one or another of the solvents were added.

TABLE III.

Tube	Amount of acetone	Lesion	Tube	Amount of acetone	Lesion
	cc.			cc.	
1	0.5	++	5	0.15	++
2	0.5	++	6	0.1	++
3	0.3	++	7	Control	++
4	0.2	++			

TABLE IV.

Tube	Amount of chloroform	Lesion	Tube	Amounts	Resultant lesion
	cc.			cc.	
1	0.2	—	4 alcohol	0.2	+++
2	0.15	—	5 toluene	0.2	+++
3	0.1	±	6 control		+++

These included chloroform, ether, 95 per cent alcohol and toluene. The materials were thoroughly mixed by means of a pipette and were incubated at 37°C. for from 75 to 90 minutes. After this the solvents were evaporated off in a vacuum and the activity of the treated virus was tested by injection into the skin of freshly shaved rabbits.

Experiment 1.—The effect of chloroform and ether in 0.2, 0.15 and 0.1 cc. amounts on 10 cc. of vaccine emulsion was tested. The mixtures were incubated at 37°C. for 65 minutes, after which the solvents were evaporated off and 0.2 cc. of the remaining virus from each tube was injected intradermally into rabbits. The results are shown in Table I.

Experiment 2.—The same procedure as in Experiment 1 was carried out except

that the mixtures were incubated for 90 minutes. The results are shown in Table II.

Experiment 3.—The action of acetone was tested by the same method as described above, the mixtures being incubated for 90 minutes. The results are shown in Table III.

Experiment 4.—Additional tests were made with chloroform, 95 per cent alcohol and toluene, the mixtures being incubated for 75 minutes. The results are given in Table IV.

The above results show that the vaccine virus is very susceptible to the action of chloroform and the action seems to grade off as the dilution is reduced. Generally 0.2 cc. will inactivate 10 cc. of the emulsion so that no lesion results from its injection. When 0.15 cc. of chloroform was used, the virus was inactive in doses of 0.2 cc. Subsequent tests have shown that when as much as 1 cc. of this virus is injected a definite but small eruption appeared in from 10 to 12 days. The injection of still larger amounts, from 5 to 10 cc. gave rise to lesions almost equivalent in severity and duration to that produced by 0.2 cc. of the untreated virus. It should be noted that there is naturally some variation in the results as the amount of the virus present in the testicular emulsion must be subject to considerable variation, as well as the natural susceptibility of the animals.

Ether, 95 per cent alcohol, acetone and toluene in amounts comparable to the amounts of chloroform found to be effective seem to have little or no action on the virus.

Reactivation of Chloroformed Virus.

With the fact demonstrated that vaccine virus is sensitive to chloroform, the next step was to determine whether the inactive or very slightly active virus could be so influenced by the addition of other agents that it would become infective in small doses. For the secondary agents, we used those found effective in activation of the chloroformed filtrates of the chicken tumor.

Preparation of Auxiliary Agents.—Chicken embryos 7 to 10 days old were obtained aseptically after careful disinfection of the shell of the egg, and were placed in tubes containing 5 cc. of Hartley's broth with glucose, potassium chloride and rabbit serum. These were incubated at 37°C. under anaerobic conditions from 4 to 25 days. After removing the tubes they were shaken in order to mix the

tissue with the fluid and were then allowed to stand or were centrifuged in order to obtain a more or less clear fluid. Sterility was tested either by smears or subcultures.

The same procedure was carried out using fragments of chicken sarcoma instead of embryos.

The third substance used in the experiments was a light suspension of kieselguhr in distilled water.

Effect of Embryo "Cultures" and Small Amounts of Chloroformed Virus.—In the first series of experiments, carried out on ten rabbits, the action was studied of a mixture of supernatant fluid of embryo "cultures" with small amounts of chloroformed virus (0.2 cc.), namely, the same amount which untreated gives rise to a typical vaccine eruption in 4 to 5 days. The results were negative as regards a vaccinal lesion. Nevertheless, the experiments brought out some interesting facts. At the end of the first 24 hours, occasionally, but generally in 2 days, there appeared a circular zone of erythema and edema, similar in dimensions to the true vaccine eruption, but more diffuse and less indurated. This increased during the 3rd day, then ceased to spread and finally disappeared more quickly than the typical vaccine eruption. Without proper control experiments, namely injection of the embryonic fluid alone, this eruption might have been the source of considerable misunderstanding. The irritating power of the embryo culture fluid proved to be weak if the cultures were young (3 to 4 days), strong if the cultures were older. The irritation is greater if the supernatant fluid to be injected contains tiny bits of embryonic tissue in suspension.

Effect of Chicken Sarcoma "Cultures" or Kieselguhr Together with Small Amounts of Chloroformed Vaccine Virus.—Some experiments were carried out with small amounts of vaccine virus, such as those mentioned above, the reactivating substance being the supernatant fluid of chicken sarcoma "cultures." These experiments were few in number, but, in general, the results justify the conclusion that these "cultures" by themselves are capable of producing in rabbits similar reactions to those produced by the supernatant fluid of embryo "cultures."

Kieselguhr produces, when injected intracutaneously, a very wide reaction, resembling the reaction caused by the vaccine virus even

more than do those produced by embryo "cultures." This point will be discussed in greater detail further on in the paper.

Effect of Embryo "Cultures" Kieselguhr Together with Larger Amounts of Chloroformed Vaccine Virus.—After the reactions induced by the embryo "cultures" had been studied and differentiated from true vaccine eruptions, the experiments were repeated with larger doses of the chloroformed vaccine and of the "culture" fluid. We further tested the activity of larger doses of the chloroformed vaccine alone keeping in mind Flu's statement that he could obtain reactivation of the chloroformed sarcoma agent only when he got tumors from the injection of large amounts of the chloroformed filtrate alone.

Experiment 1.—To 10 cc. of the vaccine virus emulsion diluted 1 to 10 with Ringer's was added 0.15 cc. of chloroform. After mixing well the tube was incubated at 37°C. for 78 minutes. The chloroform was removed in a vacuum. For the secondary substances the slightly turbid supernatant fluids from 18 day old "cultures" of embryonic tissue in Hartley's broth, and a light suspension of kieselguhr were used. The nature of the intradermal injections and the intensity of the vaccinal eruptions induced were as follows.

Rabbit 1.

		Maximum intensity of eruption
1. Chloroformed vaccine virus 0.5 cc. plus supernatant fluid embryo "culture" 0.5 cc.	Lesion +	10 days
2. Chloroformed vaccine virus 1 cc. plus supernatant fluid embryo "culture" 1 cc.	Lesion ++	10 days
3. Chloroformed vaccine virus 0.5 cc. plus light suspension kieselguhr 0.5 cc.	Lesion ++	10 days
4. Supernatant fluid embryo "culture" 0.5 cc.	No lesion	
5. Supernatant fluid embryo "culture" 1 cc.	No lesion	
6. Kieselguhr suspension 0.3 cc.	No lesion	

Rabbit 2.

1. Chloroformed vaccine virus alone 0.5 cc.	No lesion	
2. Chloroformed vaccine virus alone 0.5 cc.	No lesion	
3. Untreated vaccine virus 0.2 cc.	Lesion +++	5 days

Rabbit 3.

1. Chloroformed vaccine virus alone 5 cc.	Lesion ++	6 days
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Part of the lesion produced by the mixture of kieselguhr and chloroformed vaccine virus was removed and injected into a susceptible animal (rabbit) and a positive vaccinal eruption was obtained. All rabbits of this experiment were tested

2 months later by a second inoculation of fresh vaccine virus and found to be immune, an indication that the lesions produced were true vaccinal eruptions.

Experiment 2.—This was similar to the foregoing experiment except that 24 day old "cultures" of embryonic tissue were used.

Rabbit 1.

		<i>Maximum intensity of eruption</i>
1. Chloroformed vaccine virus 0.5 cc. plus supernatant fluid embryonic "culture" 0.5 cc.	Lesion —	
2. Chloroformed vaccine virus 1 cc. plus fluid embryonic "culture" 0.5 cc.	Lesion ++	8 days
3. Chloroformed vaccine virus 0.5 cc. plus kieselguhr suspension 0.4 cc.	Lesion ++	8 days
4. Supernatant fluid embryonic "cultures" 0.5 cc.	No lesion	
5. Supernatant fluid embryonic "cultures" 1 cc.	No lesion	

Rabbit 2.

1. Untreated vaccine virus 0.2 cc.	Lesion ++	5 days
2. Untreated vaccine virus 0.4 cc.	Lesion +++	5 days
3. Chloroformed vaccine virus 0.5 cc.	No lesion	
4. Chloroformed vaccine virus 1 cc.	No lesion	

Rabbit 3.

5. Chloroformed vaccine virus 5 cc.	Lesion ++	5 days
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The skin of the spot injected with 1 cc. of chloroformed vaccine virus alone, which did not show a definite eruption, was removed after 5 days and injected, after grinding, into the testicles of another rabbit. It gave rise to an orchitis. From this testicle a virus was obtained which gave rise to a typical pustular eruption in the skin of another animal. All rabbits of this experiment were injected 2 months later with fresh vaccine virus and found to be immune.

The above two experiments show that chloroformed vaccine virus in 0.5 and 1 cc. doses do not give rise to eruptions when injected alone into the skin of a rabbit, but the addition of the supernatant fluid of embryonic tissue "cultures" or a suspension of kieselguhr results in very definite and typical lesions. The same degree of eruption results from the injection of larger amounts of the chloroformed virus alone (5 to 10 cc.). Occasionally small amounts gave rise to eruptions so slight as to leave doubt as to their nature but for the fact that the virus may be recovered from such lesions in animal passage. The eruption which takes place as the result of the injection of a mixture

of chloroformed virus and the secondary agent, comes later than that produced by the untreated virus or the larger doses of chloroformed virus.

In the next group of experiments care was taken to test the untreated vaccine virus and the chloroformed virus on different animals in order to avoid possible immunity effects. It was thought that the reaction following the pronounced eruption from the untreated virus might interfere with a weaker delayed reaction from the treated virus.

Experiment 3.—The general procedure of this experiment was the same as the foregoing. A 39 day old "culture" of 7 day old chick embryo tissue was used as the secondary factor and the vaccine virus was exposed to the action of chloroform for 70 minutes at 37°C. The results were as follows.

Rabbit 1.

		<i>Maximum intensity of eruption</i>
1. Chloroformed vaccine virus 0.5 cc. plus embryo "culture" fluid 0.5 cc.	Lesion +	8 days
2. Same as above	Lesion ++	9 days
3. Chloroformed vaccine virus 0.5 cc. plus 0.5 cc. kieselguhr suspension	Lesion —	
4. Chloroformed vaccine virus alone 0.5 cc.	Lesion —	
5. Fluid from embryo "culture" 0.5 cc.	Lesion —	
6. Kieselguhr suspension 0.5 cc.	Lesion —	

Rabbit 2.

1. Chloroformed vaccine virus 5 cc.	Lesion ++	6 days
2. Chloroformed vaccine virus 0.5 cc.	Lesion —	
3. Chloroformed vaccine virus 0.5 cc.	Lesion —	

Rabbit 3.

1. Untreated vaccine virus 0.2 cc.	Lesion ++	5 days
2. Untreated vaccine virus 0.4 cc.	Lesion +++	5 days

One of the spots of the skin of Rabbit 2 where 0.5 cc. of chloroformed vaccine virus had been injected was removed and injected, after grinding with sand, into the testicle of a normal rabbit. An orchitis developed from which a virus was obtained which regularly gave a typical vaccinal eruption in susceptible animals.

All of the rabbits of this experiment were tested 2 months later by a second inoculation of fresh untreated vaccine virus and all proved to be immune.

Experiment 4.—The same general procedure was followed here as in the foregoing experiment except that 0.2 instead of 0.15 cc. of chloroform was added to the vaccine virus. The results were as follows.

Rabbit 1.

1. Chloroformed vaccine virus 0.5 cc. plus supernatant fluid embryonic "culture" 0.5 cc.	Lesion +
2. Chloroformed vaccine virus 0.5 cc. plus supernatant fluid embryonic "culture" 0.5 cc.	Lesion -
3. Chloroformed vaccine virus 0.5 cc. plus kieselguhr dilution 0.5 cc.	Lesion -
4. Chloroformed vaccine virus alone 0.5 cc.	Lesion -
5. Supernatant fluid embryonic "culture" 0.5 cc.	Lesion -
6. Supernatant fluid embryonic "culture" 0.5 cc.	Lesion -
7. Kieselguhr dilution alone	Lesion -

*Maximum intensity
of eruption*

8 days

Rabbit 2.

1. Chloroformed vaccine virus alone 5 cc.	Lesion -
2. Chloroformed vaccine virus alone 0.5 cc.	Lesion -
3. Chloroformed vaccine virus alone 0.5 cc.	Lesion +

8 days

Rabbit 3.

1. Untreated vaccine virus 0.2 cc.	Lesion ++
2. Untreated vaccine virus 0.4 cc.	Lesion +++

All the animals of this experiment were tested with fresh vaccine virus 2 months later and found immune.

These last two experiments indicate that the immunity possibly developing as the result of the stronger reaction to untreated vaccine did not influence the results.

The last experiment cannot be considered as entirely a satisfactory result for 0.5 cc. of chloroform vaccine alone caused a slight lesion in the control rabbit but gave a negative reaction in the test animal. The indications are that the greater the amount of chloroform used the less is the possibility of reactivating the virus. In order to get some idea of the limits the following experiments have been carried out. As the general procedure was the same and only the amount of chloroform added to the vaccine virus was varied the details of the experiment will not be gone into.

Experiment 5.—In this test 0.4 cc. of chloroform was used. Injection of as much as 2 cc. failed to produce a lesion and attempts to reactivate with "culture" fluid and kieselguhr failed to give results. The two animals developed no immunity as the result of this injection, as shown by the fact that they had an eruption 2 months later when inoculated with untreated virus.

Experiment 6.—When 0.25 cc. of chloroform was used all the injections were negative and the two animals showed no immunity on subsequent injection with fresh virus.

Experiment 7.—In this test 0.2 cc. of chloroform was used. Injection of 4 cc. of the vaccine after treatment failed to elicit a response in one rabbit and there was no reactivation by the auxiliary agent in another and no immunity developed as the result of the injection.

Experiment 8.—The above experiment with 0.2 cc. of chloroform was repeated. This time there was a slight positive eruption following the injection of chloroformed vaccine virus plus the fluid from embryonic tissue "cultures." There was also a positive result from the injection of 4.5 cc. of the treated virus alone and slight positiveness with 1 cc. and 0.5 cc. but the lesion from the latter developed some 3 or 4 days later than the lesions from the reactivated virus. All of these animals proved immune on subsequent inoculation with fresh virus.

Experiment 9.—With 0.15 cc. of chloroform the mixture of 0.5 cc. each of the treated vaccine and the supernatant fluid of the embryonic tissue "culture" gave typical eruptions. A large amount of this virus, 4.5 cc., also gave a positive result alone but smaller amounts, 0.5 to 1 cc., resulted in very slight responses. All the animals were immune on second inoculation.

Experiment 10.—Another experiment using 0.15 cc. of chloroform was carried out. In the tests the chloroformed vaccine alone even in the smaller doses gave typical eruptions. Very little difference could be made out between these and the eruptions caused by the virus plus the auxiliary fluids. All the animals were immune on second inoculation with untreated virus.

The results seem comparable to those obtained by Flu in his study of the effect of chloroform on the agent of the chicken tumor. Where no lesions result from the injection of large amounts of the treated vaccine virus it proved impossible to reactivate the smaller doses with the fluid from embryo tissue "cultures" and no immunity is developed by the animal. Our experiments show further the great variability in the eruption produced by the same dose of vaccine virus especially when one at the lower effective limit is employed. Not only do individuals differ in susceptibility but areas in the same animal differ in their response to the same dose. An extreme example of this is to be seen in the case of Rabbit 2 of Experiment 4, in which 5 cc. of the chloroformed virus failed to produce a lesion while a typical reaction occurred in another skin area receiving only 0.5 cc. While it is conceivable that errors in technique, local injury from shaving, leakage of the fluid from the puncture wound or some such factor might explain these inconsistencies, yet as far as our observations go they exist.

Localizing Effect of Auxiliary Agents.

An occasional animal of the preliminary experiments having pronounced lesions from pure vaccine showed a typical vaccinal eruption in spots injected only with fluid from an embryonic tissue "culture." This suggested a test of the secondary fluids as localizing agents when the virus was given intravenously. It will be recalled that Calmette and Guerin (13) found that the pulling out of the hair was sufficient to localize the vaccine lesion.

Experiment.—Embryonic tissue "cultures," chicken sarcoma "cultures" and light suspensions of kieselguhr were prepared in the manner already described. As a fourth substance a 10 per cent solution of peptone was injected into the skin in 0.5 to 1 cc. amounts. The supernatant fluid from the "cultures" and the kieselguhr were used in the same amounts. Five cc. of a 10 per cent suspension of vaccine virus was injected into the ear vein of the rabbits. The animals had been shaved carefully so as to avoid injury to the skin, prior to the intradermal injection of the fluids. The resultant eruptions were tested by removing the skin area, grinding and injecting into the skin of a fresh animal, and only these eruptions proving to have active virus by this test were included as positives.

In the experiment proper eight animals were injected in five or six different spots with the fluids prepared as described above, followed by the intravenous injection of the vaccine virus. In addition seven rabbits were used to test the presence of active virus in the resultant lesions. The results are given in Table V.

TABLE V.

	Spots injected	Positive localization	Doubtful localization	Negative localization	
Embryonic "cultures"	29	25	1	3	The 3 negative cases belong to a rabbit injected with a very clear supernatant fluid
Chicken sarcoma "cultures"	9	6	—	3	
Peptone	4	—	—	4	
Kieselguhr	6	—	—	6	

The above results suggest that the action of the secondary fluid is not on the virus but upon the cells rendering them more susceptible to its action. While the number of tests with kieselguhr is small the

failure of the virus to localize in a single instance in the area of reaction produced by this agent is significant. It is of interest in this connection to note that the agent of the chicken tumor on intravenous injection shows less tendency to localize in kieselguhr reactions than in the reactions produced by the several other substances (14).

DISCUSSION.

It was the purpose of these experiments to parallel the work of Gye, but with vaccine virus instead of the chicken tumor agent. In planning of the tests, we took into consideration the critical work of Murphy and of Flu as well as the various results obtained by several other investigators who have attempted to repeat Gye's experiments. While the vaccine virus has certain advantages in a study of this kind, the chicken tumor agent has a special one in that natural resistance against it is so feeble that a tumor once started rarely fails to progress. In spite of the variability in the reactions reported in this paper, sufficient data of a positive nature are provided to justify certain conclusions.

Flu states that an auxiliary substance will render a small amount of the chloroformed chicken tumor filtrate infective only when a large dose of the chloroformed filtrate alone is capable of inducing a tumor. The evidence brought out by the present experiments indicates that the same is true of the vaccine virus. When the amount of chloroform used was large enough to render even great amounts of the virus innocuous no reactivation of the small dose proved possible. It is of interest to note that about the same amount of chloroform is required to inactivate the virus and the tumor agent. It would seem from these results that chloroform in amounts which still allow reactivation of the virus does not destroy all of the agent. One may suppose either that the number of infective elements is greatly reduced in number or that the infectivity of all of the elements is lowered by the chloroform treatment.

The secondary substances or activators would seem to exert their effect by rendering the animal's cells more susceptible to the infecting power of the virus. This is indicated by the result of the experiments in which it was demonstrated that the fluids most effective in reac-

tivating the chloroformed virus will induce a reaction in the skin that is favorable to localization of virus injected intravenously. The fact that some substances are more active than others in this respect regardless of the amount of injury induced, opens up an interesting question as to the type of injury or reaction which determines the localization of an infective agent. This point would bear further investigation not only as concerns the vaccine virus but the chicken tumor agent as well.

The analogy between these results and those obtained by Gye with the chicken tumor agent would tend to uphold the conclusions of Murphy and of Flu that the chloroform treatment attenuates the agent but does not destroy it; and the secondary factor contained in the "cultures" is non-specific in its action, merely rendering the cells of the inoculated animal more susceptible to the enfeebled agent.

Summary and Conclusions.

Vaccine virus, obtained from testicular inoculation shows a high susceptibility to chloroform as compared with ether, toluene, 95 per cent alcohol and acetone.

Vaccine virus, after treatment with an amount of chloroform sufficient to render it incapable or only barely capable of originating an eruption in the rabbit's skin, produces a characteristic eruption when injected with the supernatant fluid of embryonic tissue or sarcoma tissue "cultures" or kieselguhr, substances all of which are markedly irritative to the rabbit's skin.

Reactivation of the chloroformed vaccine virus is not possible when chloroform has been added to it in such quantity that the injection of large amounts of the treated virus fails to cause an eruption. Whenever reactivation has been accomplished it has been possible to get a vaccine eruption of greater or less intensity by the injection of large amounts of the chloroformed vaccine alone.

Embryo and chicken sarcoma "culture" fluids when injected intradermally make the skin susceptible to the localization of the virus introduced intravenously.

The bearing of these experiments on the interpretation of Gye's theory of cancer causation is discussed.

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A STUDY OF THE MECHANISM OF NUCLEINATE-INDUCED
LEUCOPENIC AND LEUCOCYTIC STATES, WITH
SPECIAL REFERENCE TO THE RELATIVE
RÔLES OF LIVER, SPLEEN, AND
BONE MARROW.

BY CHARLES A. DOAN, M.D., LEON G. ZERFAS, M.D., SYLVIA WARREN,
AND OLIVIA AMES.

*(From the Thorndike Memorial Laboratory, Boston City Hospital, and the Department
of Medicine, Harvard Medical School, Boston, and the Laboratories of
The Rockefeller Institute for Medical Research, New York.)*

PLATES 21 AND 22.

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The newer concept that there is normally a wide range of fluctuation in the total number of circulating red and white cells (1-5), reopens the whole question of the meaning of variations in blood counts. To many conditions, such as digestion and exercise, and to many chemical substances have been attributed specific changes in the blood count, which now are known to fall within the more recently established limits of the normal. Hence, in distinguishing between the delivery of new cells from marrow or lymph glands, and changes in distribution of cells already within the circulation, it becomes necessary to study all the factors that might be involved. Each particular stimulus under consideration must be known with reference to its particular point and mode of activity. As will be demonstrated in this paper the subject can only be analyzed in the living animal, for the position of cells in the blood vessels after death is no indication of their distribution during life. In the problem it is essential to study the influence of vasomotor reactions on the distribution of blood cells, to consider possible changes in blood volume, and especially to analyze the rôle of various organs on the peripheral concentration of the cells.

The observation by Sabin (6), in a survey of normal living blood cells, of certain "non-motile" polymorphonuclear neutrophils, and the

later determination (1), of their occurrence in showers in the peripheral blood, led to the hypothesis that the effective normal stimulus might be the liberated products from disintegrating cells. This could occur either directly or through the medium of the phagocytic group of cells reducing the debris. The correlation of the showers of "non-motile" cells in human pathological conditions with a subsequent increase of young motile neutrophils (7) further supported the concept. This seemed to point to a thorough restudy of the effect of nucleic acid and its derivatives on the leucopoietic system, inasmuch as these substances must be constantly produced physiologically in the body.

Nucleic acid was first isolated by Altmann (1877) from Miescher's nuclein. Miescher worked chiefly with pus, so readily obtained in those days of septic surgery, and since pus consisted mostly of nuclei, and his minimal residue was cytoplasmic, he reasoned that the material he obtained, which was high in phosphorus content, came from the nucleus, and hence the name "nuclein" (8).

Following the emphasis placed by Metchnikoff (9) on the "phagocytic power" of the polymorphonuclear leucocytes as the *modus operandi* for destroying bacteria, Ames and Huntley (10) were among the first to study nucleic acid experimentally from the standpoint of its apparent production of a leucocytosis. They concluded from the hypodermic injection of nuclein solution into dogs that there was an increase in the number of leucocytes in the central and peripheral circulation, and, further, since they found an increase of young cells, that the response was a true delivery of cells from the bone marrow. Milroy and Malcolm (11), using the sodium salts of yeast and thymus nucleic acid and their decomposition products, thymic acid, adenine, guanine, and cystosine, found after subcutaneous injections in rabbits and guinea pigs what they interpreted as a specific hyperleucocytosis, but the increases were from 5500 to 6800, or from 6000 to 7500, a range of variation hardly permissible of such interpretation today.

The first advocacy of nuclein therapy was on purely theoretical grounds, the claim being made, for instance by Vaughan, Novy, and McClintock (12), that by its use the germicidal power of the blood could be increased and hence the resistance heightened to diseases of microbic origin. However, the effectiveness of nucleic acid, in certain clinical conditions, through its apparent stimulation of an increase in the white count, has been periodically suggested by both physicians and surgeons (13-18), though the increases in white cells, which have been reported, are equivocal, and, for the most part, fall within the physiological range for the white count. Thus Habetin (19), in 1923, advocated the injection of 10 cc. of a 5 per cent solution of sodium nucleinate subcutaneously as a clinical test of bone marrow function, describing the reaction in healthy individuals as an increase of from 40 to 60 per cent of cells, whereas, as is now known, the normal range of

fluctuation is 100 per cent in 24 hours (1, 2). Larsell, Jones, Nokes, and Phillips (20, 21) recently reported an experimental and clinical study of the hemopoietic effects of intravenously injected nucleic acids. In rabbits they found hemopoiesis stimulated by the nucleic acid from the nuclei of bird's cells, and in selected human cases of anemia they describe a comparable but temporary hemopoietic stimulation. Robertson, Hicks, and Marston (22), on the other hand, found no leucocytosis, either relative or absolute, in human subjects on an otherwise purine-free diet, following the oral ingestion of nucleic acids of vegetable or animal origin.

Löwit (23) ascribed the "leucopenia" he found following injections of peptone, nucleins, and sodium urate to a leucolytic phenomenon in the inner organs; the leucocytosis which followed he thought a function of this lysis reflected in a direct response from the bone marrow. These leucopenic-leucocytic fluctuations of the white cells have been the subject of study and conjecture ever since, the mechanism having been in turn attributed to lysis (23), redistribution phenomena (24, 25, 26), positive and negative chemotaxis either within the circulation only (27) or between circulation and marrow.

That additional data relative to the fundamental mechanism of the changes ascribed to nucleinate might be available, the series of experimental observations here reported were begun in the fall of 1924.

Technical Detail.

Sodium nucleinate (Merck) from yeast nucleic acid was made up in fresh, glass-distilled water so that each cc. contained 100 mg. There was no essential difference in the action of two different lots from this source nor in a sample obtained from Dr. Henry Jackson, Jr. The biuret reaction for protein was negative when the test was applied to the sodium nucleinate of these experiments. The adenine and guanine nucleotides first used were prepared by the autoclave method of Jones (28) and made available also through the kindness of Dr. Jackson of the Thorndike Laboratory. Chemically pure, crystalline adenylic and guanylic acids were later supplied through the generosity of Dr. P. A. Levene of The Rockefeller Institute, and used with results entirely comparable in every respect with those obtained from the nucleotide products from the first source. The solutions before injection were brought to pH 7.8 by the addition of $N/10$ sodium hydroxide. A relatively large dosage was employed, in the majority of instances 1 gm. in 10 cc., in order that the effects, if any, might be decided enough for detection and analysis of the mechanism. Clinically there was considerable variation in the severity of the reaction in rabbits to the intravenous injections of 1 gm. of the sodium nucleinate, a more or less marked vasoconstriction occurring for from 15 minutes to $\frac{1}{2}$ hour, followed by vasodilatation and transitory diarrhea; the temperature registered at times 105°F. (normal for rabbits 102–104°F). The injection of 2 gm. of sodium nucleinate intravenously in Cat 1, respiratory and myocardiographic tracings be-

ing recorded on the kymograph, showed a very transitory increase in the rate, but not in the amplitude of the respirations, and a moderate rise in the blood pressure maintained for a somewhat longer period. As the nucleotides are less soluble than the nucleinate in distilled water and tend to form a gel if the proper pH is not maintained, care is necessary in their preparation for intravenous use. However, given in the proper dilutions the guanine and adenine nucleotides did not produce the clinical symptoms of toxicity and fever noted with sodium nucleinate in certain animals.

All counts were made with Bureau of Standards equipment. The hemoglobins were read in a Duboscq colorimeter with the Newcomer standard. The hematocrit determinations were made with the Van Allen pipette. Refractometric readings were made for the serum protein estimations. For the Armeth differential counts cover-slip preparations were stained with Wright's blood stain and 100 cells counted from each slip and the average percentages taken. In some of the experiments the differential counts of the white cells were made with the supravital technic (6) using vital neutral red. The usual routine was employed in the taking of the various consecutive samples (7).

In the experiments in which comparable observations were made from ear vein, heart, and liver, the preparations were taken by three people simultaneously. Through the anterior mediastinum a needle was introduced into the right ventricle; at the same time a small triangle of liver tissue was removed through a small abdominal incision and fixed at once in Helly's fluid for histological study, the count being taken from the blood welling forth from the freshly cut surface. A warm sponge controlled the hemorrhage from the liver, and each succeeding sample was taken from a fresh area far enough removed (2 cm.) so that the former manipulation could not affect the local concentration of white cells. Similar consecutive samples of the spleen were taken for fixation and studied histologically in series, total counts from the splenic pulp being impossible for comparative studies. All such experiments were performed under general anesthesia with sodium-barbital, 0.4 gm. per kilo being injected intravenously prior to operation. Repeated control counts from the peripheral blood were taken before and after the sodium-barbital and during the operative procedures, in order to establish their influence on the cellular equilibrium of the individual animal before any special substances were introduced.

The rabbits undergoing splenectomy were operated upon with aseptic surgical technic, under ether anesthesia, and peripheral blood counts as well as other clinical observations were used as an index of their condition before special procedures were undertaken. In only one instance was appreciable leucocytosis noted following, and apparently related to, splenectomy.

Through the courtesy of Dr. Joseph T. Wearn, it was possible to carry out experiments with the oncometer in which changes in the volume of the spleen, during the reaction to sodium nucleinate, were recorded on the kymograph. Because of the small calibre of the vessels entering and leaving the spleen in the rabbit, it is difficult to secure excursions of systole and diastole, such, for example,

as are observed with the kidney; nevertheless, the pulsations of the arteries entering the spleen were plainly visible through the glass oncometer at all times, the return flow through the veins was unobstructed, a constant temperature was maintained, and the tracings recorded were in every particular identical with those reproduced by Roy (29), Schäfer and Moore (30), and others. Therefore, it is believed that these observations may be taken as at least tending to support the general conclusions with reference to the spleen, that have been reached by the other avenues of approach to the problem.

Forty-one rabbits were studied. Many of them were used for repeated experiments for comparison and contrast of the same and different substances in the same individuals, as may be seen from the charts. Six experiments were performed, in which a survey of peripheral blood, heart, and liver after sodium nucleinate was made every 15 minutes, repeated samples of liver and spleen being removed for histological study. Uncomplicated studies of the peripheral blood after sodium nucleinate were made in six rabbits, after guanine nucleotide in six rabbits, and after adenine nucleotide in three. Splenectomy was performed in seven rabbits; and tracings with the spleen in the oncometer were taken in ten animals to correlate with cellular data, from the peripheral blood, after sodium nucleinate. Correlation of repeated differential counts from peripheral blood and splenic puncture, taken before and after sodium nucleinate, was made in two animals (Table I).

The Bone Marrow during the Reaction to Sodium Nucleinate.

Following the intravenous administration of 1 gm. of sodium nucleinate into normal rabbits, there developed immediately in practically every instance a leucopenia which lasted for several hours (Charts 1 to 3 A). No leucolytic effect could be observed in *in vitro* tests made according to a method previously described (31) and the transitory vasomotor disturbances in the peripheral vessels passed off much sooner than the leucopenia. A leucocytosis, usually of considerable magnitude (50,000 to 100,000), but varying with the individual, succeeded this leucopenic period and in some instances lasted into the 2nd, 3rd, and 4th days (Chart 1). During the leucopenia the fall in total number of cells was primarily due to a decrease in the neutrophilic group (Chart 3), while the leucocytosis reflected greatly increased numbers of this cell type. The basophilic and eosinophilic leucocytes followed the fluctuations of the neutrophilic cells in miniature, disappearing entirely in the leucopenic phase and showing a slight increase in absolute number during the leucocytosis. The lymphocytes, in our experience, may or may not decrease in

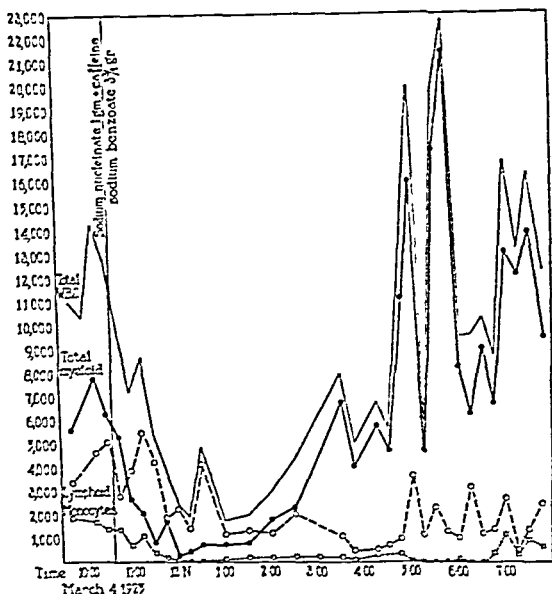


CHART 3A. Rabbit 3, T 35. There is here a depression of lymphocytes and monocytes following sodium nucleinate, but with only a return to normal thereafter, and some animals have not shown the depression in these groups. Both leucopenia and leucocytosis are primarily a reflection of variations in the myeloid group of cells originating in the bone marrow. Differential count of white cells made from cover slip films stained with Wright's blood stain.

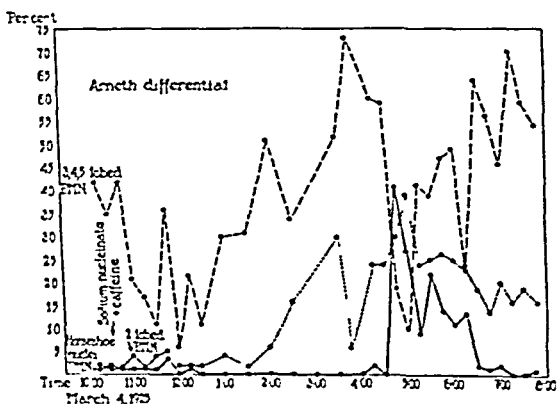


CHART 3B. Rabbit 3, T 35. There is a "shift to the left" in the Arneith pattern first noted 4 hours after sodium nucleinate and coincident with the onset of leucocytosis. This "shift" is indicative of the large part played by the bone marrow in the reaction.

absolute number during the leucopenia, but we have never observed a subsequent lymphocytosis of any moment in this series. The same may be said of the monocytes. A "shift to the left" in the Arneth pattern (Charts 3B, 10B) of the nuclei in the polymorphonuclear neutrophilic leucocytes indicated an increased delivery of cells from the bone marrow as at least one factor of importance in the phenomenon. Histological examination of the bone marrow during the hours just following the sodium nucleinate showed a markedly speeded up process of maturation of Myelocytes "C" (32, 33) into motile leucocytes; a mobilization of these cells about patent sinuses together with active diapedesis characterized the marrow in every instance.

Fig. 1 is a photomicrograph of the marrow from Rabbit 6 (Chart 6) 2 hours after the injection of 1 gm. of sodium nucleinate, while the period of leucopenia still existed in the general circulation, as indicated by counts from liver, right ventricle, and periphery. The sinus is surrounded by a continuous border of encroaching neutrophilic leucocytes in active diapedesis, the open spaces to right and left indicating the gradual depletion of the myeloid reserve of that focus. This appearance is identical with that observed after the injection of inactivated typhoid organisms (Figs. 22 to 24 in a former paper (34)). Fig. 2 shows a marrow after two injections of sodium nucleinate, the second having been given on the 4th day following the first, when the count had regained the normal level. The depletion of the myeloid elements with the beginning displacement of the fat cells preliminary to reparative metaplasia reveals a latent response on the part of the marrow, similar to that when a demand is made for increased cells from the late myelocyte level without the accompanying presence of a maturation factor (33, 35).

Analysis seemed to indicate that the response from the bone marrow to nucleinate preceded the subsidence of the leucopenia. To measure the response and to find if possible the location of the white cells during the leucopenic phase, surveys of certain of the abdominal viscera were undertaken.

The Liver and the Lungs during the Reaction to Sodium Nucleinate.

In none of the experiments was there observed a rise in the cells in the liver during a fall in the peripheral blood, while the circulation of the animal was maintained unimpaired.

Charts 4 to 6 give the concentration of white cells in ear vein, right heart, and liver with the animal under sodium-barbital anesthesia and the abdomen opened

under aseptic precautions. In each instance the leucopenia following sodium nucleinate was registered in the right ventricle and liver as well as in the peripheral blood, with no gross evidence of dilatation of the vessels of the splanchnic area. In both Rabbits 5 (Chart 5) and 6 (Chart 6) the counts in the peripheral blood

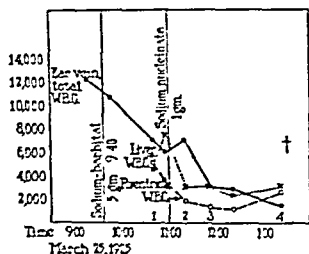


CHART 4.

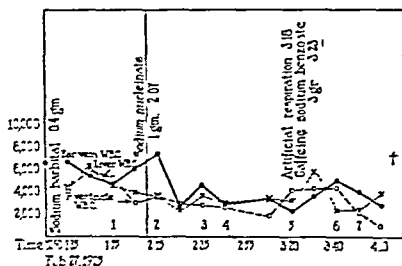


CHART 5.

CHART 4. Rabbit 4, T 49. The leucopenia following sodium nucleinate is the expression of a depression in the total white cells in the general circulation (counts from peripheral vein, right ventricle, and liver). The situation of the digits along the abscissa in Charts 4, 5, and 6 indicates the points at which liver and spleen tissue were removed for histological study.

CHART 5. Rabbit 5, T 37. The changes in total white cells following sodium nucleinate, the institution of artificial respiration, and the administration of caffeine are similar; specimens from periphery, right ventricle, and liver.

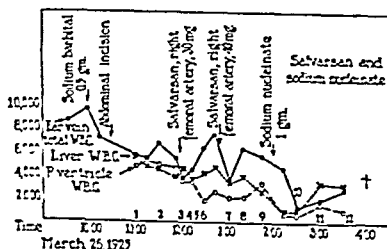


CHART 6. Rabbit 6, T 50. The leucopenia after salvarsan or sodium nucleinate occurs both in the central and peripheral blood; successive simultaneous counts from ear vein, right heart, and liver.

were higher, not lower than the other counts taken simultaneously, but the general trend of the total counts was in the same direction. Two injections of salvarsan in Rabbit 6 gave temporary depressions in the total cells without an increase in cells in the liver, though the transitory leucopenia following such injections has been attributed to this organ (36). The figures along the abscissa in each chart

indicate the points at which tissues from spleen and liver were removed for fixation. The microscopic study of sections of the liver from all the animals of the series showed that there had been no accumulation of leucocytes in the capillary bed of the organ during leucopenia.

Most of the estimations of the local concentration of cells, heretofore, have been made from histological sections of postmortem material or from counts from the liver after the circulation had ceased. In following successive counts from the liver both before and after death the explanation may be seen, perhaps, for the conception, generally current, of the large part played by the liver as a storehouse for the white cells in leucopenia. The profound disturbance in distribution of cells in the circulation at the moment of death makes it essential to take all preparations for total and differential counts of blood cells from organs while the animal is still alive and in good condition, that is to say, not in a state of shock.

In Chart 7 it will be seen that during the leucopenia the white cells in the liver remained low and only began to rise gradually coincident with the rise of cells in the general circulation. However, within 2 minutes of the death of the animal, the total count of white cells in the liver had risen from 11,000 to 37,000; the differential counts of the white cells, on the other hand, at 4:20 and at 4:24 were identical, a finding quite at variance with the increase as analyzed in the peripheral blood during the leucocytosis following sodium nucleinate (Chart 3). In this animal red cell counts were taken from periphery and liver at the same time that the white counts were being noted, in order that any changes in blood volume might be indirectly estimated. The remarkable constancy of the variations in the number of red cells in liver and periphery shows the specificity of the fluctuations in white blood cells.

Rabbit 8 (T 67) was splenectomized. The counts in liver and periphery after sodium nucleinate were 5200 and 8800 respectively. During the experiment the animal died and 2 minutes after death the white cells in the liver were 23,700 per c.mm. In this instance also red cells from liver and ear vein were taken, as in Rabbit 7, and showed the same gradual fall in total numbers per cc. In Rabbit 15 (T 101), uninjected and normal, the total count from the liver just prior to death by air embolism was 6300, 1 minute after death it was 10,300, and 6 minutes after, 19,000. Rabbit 16 (T 91) showed 8000 cells in the liver just before death by air embolism, at the end of an experiment with the spleen in an oncometer during sodium nucleinate leucopenia and leucocytosis, while 2 minutes after death the total white cells in the same organ were 24,000 per c.mm.

Thus, whether under normal conditions, or in the splenectomized animal, whether during the height of the leucopenia or during the

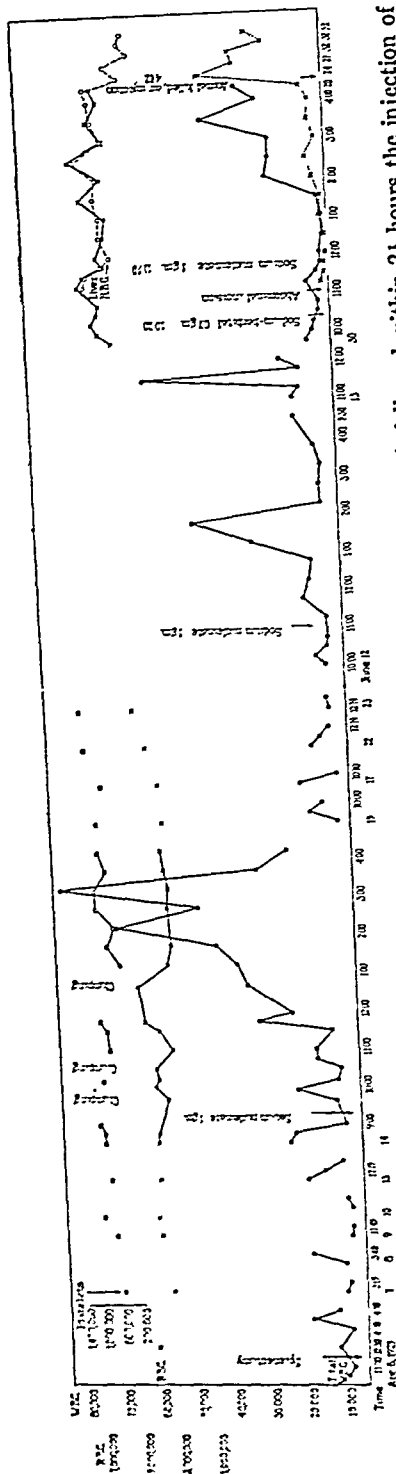


CHART 7. Rabbit 7, T 54. On three successive occasions over a period of 3 months leucocytosis followed within 2½ hours the injection of 1 gm. of sodium nucleinate into the splenectomized rabbit. Platelets and red cells are charted for comparison. On the last occasion red and white cells from liver and periphery were counted and the experiment terminated by air embolism. The immediate change in the concentration of the white cells in the liver after cessation of the circulation is graphically recorded.

leucocytosis after sodium nucleinate, consecutive counts taken from the liver before and after death show the same marked relative discrepancy in total numbers, though not in differential counts. In other words, the sudden increase in total white cells in the liver immediately after death is due to a redistribution of the white cells in the circulation at the moment of death, and not to a sudden influx of new cells from the bone marrow or spleen, as when a true increase in available cells of any type occurs. It would seem, therefore, that all estimates of redistribution phenomena must be made with the circulation unimpaired, and that, under the conditions of the experiments here cited, the changes within the liver blood do not initiate but reflect the leucocytosis following leucopenia after sodium nucleinate and salvarsan. There is within the organ no concentration of cells during the period of depression other than that found in the general circulation. All sections studied in this series showing increased numbers of granulocytes in the capillaries of the liver were taken at a time when there was a leucocytosis in the peripheral blood also. All tissue was taken from the anesthetized living animal and fixed immediately.

Since these observations were made, A. F. B. Shaw (2) and Webb (37) have reported similar studies on leucocytic distribution. Shaw found in normal rabbits and dogs under chlorotone a uniform distribution of leucocytes at any given moment throughout the body, *viz.*, periphery, brain, liver, kidney, lung, mesenteric vein, and both sides of the heart. He emphasized the dangers in prolonged experiments of maldistribution of the leucocytes, induced, presumably, by circulatory disturbances following shock, and reported profound inequality in body distribution of leucocytes immediately after death. The problem of leucocytic distribution in the dog's body during horse serum anaphylaxis was studied by Webb, who found the transitory decrease in total cells from the capillary blood of liver, intestines, kidney, spleen, bone marrow, and from portal vein to parallel that of the periphery. This was controlled by the study of sections which revealed no aggregations of leucocytes within the capillaries of these organs. However, from the pulmonary vein during acute anaphylactic shock he obtained fewer leucocytes than from the pulmonary artery, and sections showed enormous numbers of leucocytes in the capillaries of the lungs. This confirms the earlier findings for the lung of Goldscheider and Jacob (24), Bruce (38), Andrewes (39), and others working with protein and bacterial antigens. However, Schilling (40), in studying Oeller's (41) tissues after anaphylaxis, analyzed this finding of specific accumulations of leucocytes in capillaries of the lung as secondary to the enormous local swelling of the endothelial cells. This would seem, therefore, to be a special

phenomenon associated with anaphylactoid sensitivity, the leucopenia being secondary to the mechanical factors involved in local swelling of the endothelium of the pulmonary capillaries. On the other hand, a study of the lungs during the leucopenic phase after sodium nucleinate, or as Wells (42) found after bacterial injections, showed no such accumulations of leucocytes to account for the peripheral leucopenia. Wells studied the leucopenia in rabbits following bacterial injections, and reported the finding of uniformly low counts from the blood of splenic and mesenteric arteries, splenic, portal, hepatic, and superior mesenteric veins, the parenchyma of the lung, both ventricles of the heart, and the marginal ear vein. In sharp contrast were the number of leucocytes obtained from bone marrow and liver, and especially from the blood of the parenchyma of the spleen.

TABLE I.

Relationship of Myeloid Cells in Peripheral Blood and Spleen Following Sodium Nucleinate.

Rabbit No.	Time	W.B.C. peripheral blood				Spleen puncture			
		Total	Myeloid	Lymphoid	Monocytes	Myeloid	Lymphoid	Monocytes	Chasmatocytes
17 T98	Before Na nucleinate	7000	66	24	10	19	78	2	1
	1½ hrs. after Na nucleinate	6000	47	45	8	40	56	4	0
18 T99	Before Na nucleinate	18000	64	31	5	16	78	1.5	4.5
	1 hr. after Na nucleinate	8400	30	68	2	30	66	2	2
	2½ hrs. after Na nucleinate	35000	82	14	4	48	48	1	3

The Spleen during the Reaction to Sodium Nucleinate.

Having demonstrated that the leucopenia was not due to a stasis of leucocytes in the liver, lungs, or splanchnic area we turned more directly to the spleen as the organ of all the abdominal viscera most likely to be involved in the general phenomena in which we were interested, both from the nature of its anatomical structure and from what is known of its function. Because of the large number of cells in the spleen not in circulation at any given moment, total counts to compare with those from heart and liver were not attempted, but instead the differential count of cells, obtained by puncture with a fine capillary pipette, was made with the supravital technic (43), and compared with the findings in the peripheral blood. With this method

successive splenic punctures during the leucopenic-leucocytic periods following sodium nucleinate showed a gradual but definite increase in percentage of neutrophils in the splenic parenchyma.

It will be seen from Table I that the myeloid cells may be considered to make up from 15 to 20 per cent of the cells obtained under the conditions of splenic puncture. In Rabbit 17 (T 98), 1½ hours after sodium nucleinate, they had increased 21 per cent, or to 40 per cent of all the cells, while the myeloid cells in the peripheral blood had decreased from 66 to 47 per cent. In Rabbit 18 (T 99) the increase of myeloid cells in the spleen was from 16 to 30 per cent in 1 hour after sodium nucleinate, and to 48 per cent in 2½ hours, while the peripheral count fell from 18,000 to 8400 and myeloid cells from 64 to 30 per cent the 1st hour. The total number of lymphocytes in the peripheral blood throughout remained relatively constant though showing percentage fluctuations of 31 to 68 to 14 per cent, again emphasizing the specificity of the effect of the nucleinate on the neutrophilic group of white cells.

The histological evidence from the fixed sections of spleen, removed in series at the times when the specimens of the liver, previously mentioned, were taken, shows a gradual increase in neutrophilic leucocytes in the parenchyma of the spleen following sodium nucleinate.

In Charts 4 to 6 the points at which splenic tissue was removed for section are indicated by the digits along the abscissæ. Fig. 3 represents the normal spleen of Rabbit 4. Fig. 4 shows another portion of the same spleen 1 hour after sodium nucleinate (see Points 1 and 3 as marked on Chart 4). The beginning accumulation of small foci of granulocytes in Fig. 4, more particularly, at this early stage after the nucleinate, near the vessels, may be seen. The diffuse scattering of increased numbers of individual neutrophils throughout the parenchyma of the organ is not so apparent at this magnification. Fig. 5 is a photomicrograph of a section of the spleen of Rabbit 19 (T 31), 8 hours after sodium nucleinate during a leucocytosis reaching 60,000 to 70,000, which had started 3 hours before, after a leucopenic period of 5 hours.

This is representative of several experiments tending to show that the leucocytosis following the leucopenia is not simply the releasing of the cells stored up during the leucopenic period, in other words a temporary redistribution phenomenon, but the result instead of an increased activity of the hemopoietic centers, initiated early and maintained for considerable periods.

The series of oncometer experiments for the study of changes in volume of the spleen during the leucopenia-leucocytosis reaction to sodium nucleinate reveal a definite increase in the size of this organ.

The kymograph records, covering periods of hours, are far too voluminous to reproduce except in very schematic form, but in all particulars they resemble those illustrated in similar studies on the spleen (29, 30). The relative changes in splenic volume, as recorded and synchronized with the total white count from the peripheral blood, are represented diagrammatically in Chart 15. The control period gave the well recognized inherent contractions of the spleen during the base line observations 45 minutes before 1 gm. of sodium nucleinate was injected into the ear vein. Coincident with the onset of leucopenia, the splenic volume started to increase; when the count first began to rise (5.25 p.m.), there was a slight contraction of the spleen, but as the count reached 50,000, the volume again increased, reaching an equilibrium. At a moment when the total count had fallen again to about 10,000, 4 cc. of a 1:1000 solution of adrenalin was introduced into the vein; this was followed immediately by a strong contraction of the spleen and a coincident rise in the white count to 26,400; after the return to the former volume and white count, mechanical stimulation of the saphenous nerve gave a similar synchronous fall in spleen volume and rise in peripheral white count, differing only quantitatively from that previously recorded.

Roy (29), who first adapted the plethysmograph to the investigation of changes in organ volume, described the rhythmic contractions of the normal spleen in dog, cat, and rabbit, and found that stimulation of the central end of a cut sensory nerve caused a rapid contraction of the spleen. Oliver and Schäfer (44) and Schäfer and Moore (30) confirmed this and described an enormous contraction of the spleen after the injection of extracts of the suprarenal gland.

Apparently in a state when the spleen is known to contain large numbers of myeloid cells, as illustrated in the case of Rabbit 19 (Fig. 5), any marked contraction of the organ might be expected to force out neutrophils, even as it does lymphocytes, under adrenalin stimulation in the normal (7). Barcroft's (45) conclusions with reference to the relation of spleen and the reserve of red cells would seem equally apropos for the white cells under the conditions just cited: "It (the spleen) has a function entirely in conformity with its muscular structure, being in fact a reservoir of corpuscles at once fitted by its reticulum to detain them, and by its musculature to expel them when required to do so." de Boer and Carroll (46) further deduced that the material expressed from the spleen was largely corpuscles (red cells) because the CO content of the blood in the splenic vein drawn during a contraction was intermediate between that of splenic artery and pulp.

A study of the platelets in the majority of the experiments here cited (Charts 7, 9, 14) revealed a clumping into large masses quite

impossible to separate for counting, during the period represented by the leucopenic phase. In the spleen an increase in the number of granulocytes phagocytized within the clasmatocytes was noted during their increasing infiltration of the parenchyma. These observations suggest that the nucleinate molecule in some way alters temporarily the surface of these cells, and of the platelets, sufficiently to permit of their adhesion and filtration in the parenchyma of the spleen, but not within the endothelial lined capillary beds of other organs. Those sufficiently injured are taken up by the phagocytic cells so abundant in the spleen normally, the remainder being returned to the circulation at a later time. That the majority of the white cells are not permanently injured or destroyed seems clear. Also the fact that lymphocytes and monocytes remain relatively undisturbed in many of the reactions militates against the concept of a purely mechanical factor in the leucopenia.

The Effect of Splenectomy on the Leucopenia Produced by Sodium Nucleinate.

The crucial test of the importance and extent of the rôle of the spleen in the leucopenia of sodium nucleinate origin is obviously to be found in observations after splenectomy. A series of splenectomies were accordingly performed before, during, and after sodium nucleinate administration.

No depression or shock, such as that described by Larsell, Nokes, and Phillips (20), was noted in the splenectomized rabbits after nucleic acid. Chart 8 shows the leucopenia following 1 gm. of sodium nucleinate with the removal of the spleen at the time which, in the majority of the animals, proved to be about the middle of the period of depression in the peripheral count. A leucocytosis reaching 52,000 was immediately precipitated. It will be seen from Charts 7, 9, and 10 A that aseptic splenectomy does not cause such a rise in the white count *per se*.

Partial splenectomy, as performed in Rabbit 9 (Chart 9), did not prevent the usual leucopenia, but after complete removal of the remaining portion of the organ at a later operation there was not the characteristic leucopenia with sodium nucleinate in the original dosage, and the leucocytosis followed more speedily, the highest count reaching 110,000. In this instance the lowered platelet count was due to the agglutination of the platelets and not to an actual decrease in their numbers, and the red counts indicate indirectly the relative constancy of the blood volume. Rabbit 7 (Chart 7) showed the onset of leucocytosis after splenectomy

within 2½ hours of the sodium nucleinate injection on three different occasions over a period of 3 months. On all three occasions the tendency of the white count to maintain its normal rhythmic equilibrium undisturbed until the real increase was apparent from the bone marrow was clear from the preliminary counts, which may be taken as an indication that the latent period of response for the hemopoietic organs under this stimulus in this individual was between 2 and 3 hours. Here again the clumping of the platelets was found but without an accompanying leucopenia as in the normal rabbits, and the close conformity of red cells from marginal ear vein and liver indirectly controls the estimation of blood volume and stasis factors.

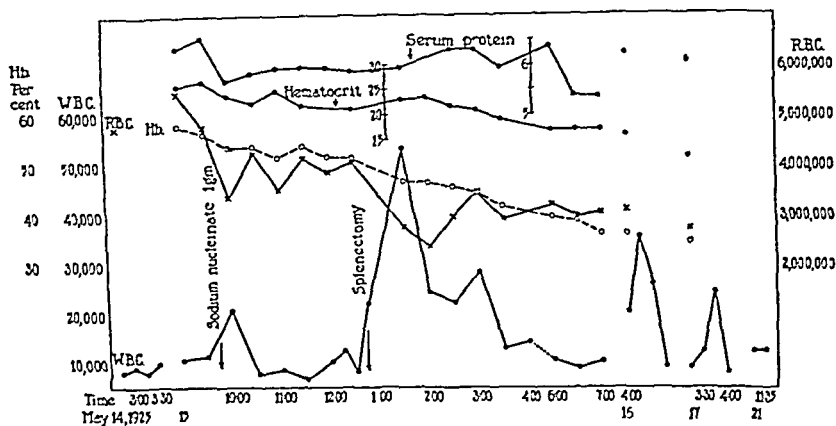
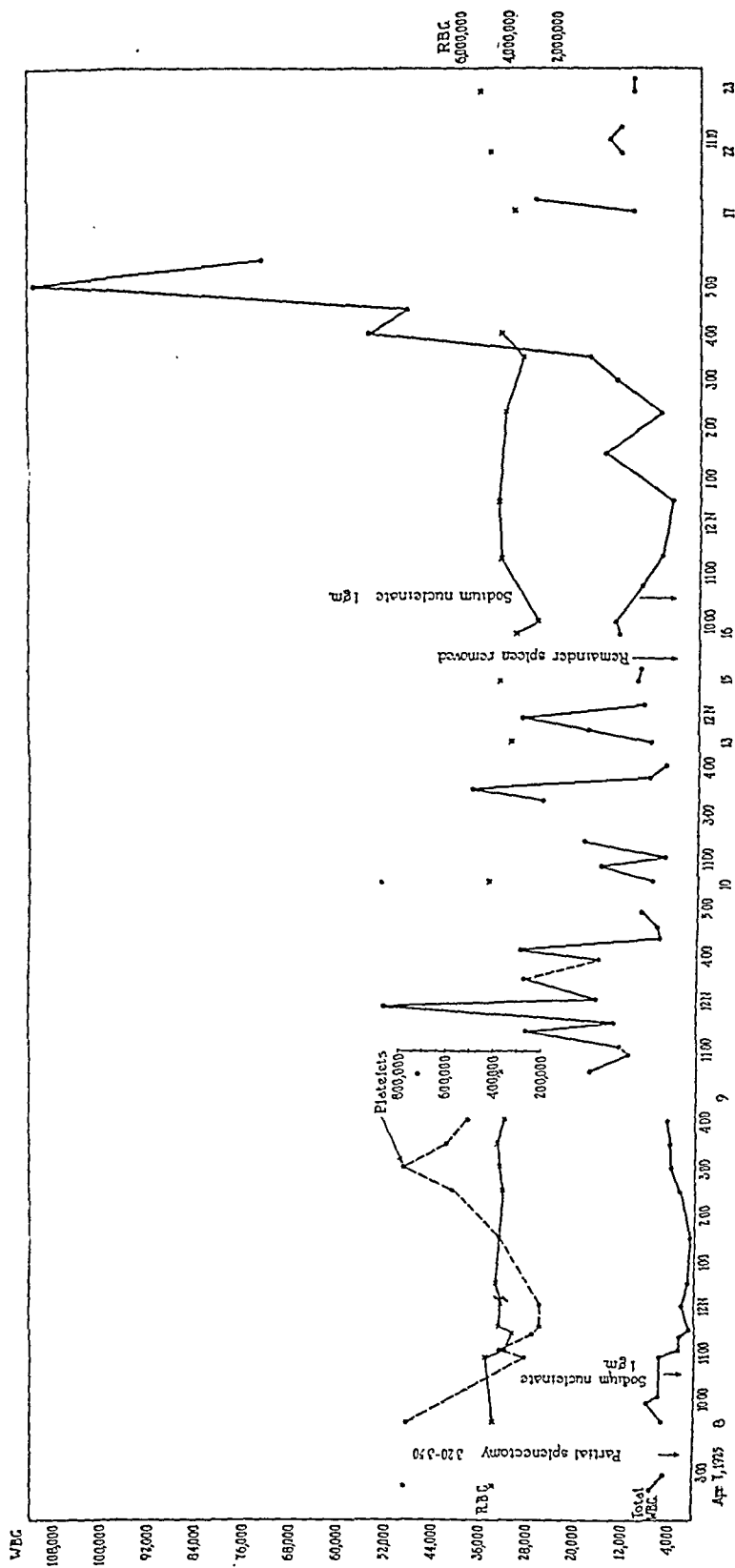


CHART 8. Rabbit 8, T 67. Splenectomy in the midst of the leucopenic period after sodium nucleinate was followed immediately by a leucocytosis reaching 55,000, a high zonal range for the white cells being maintained on the succeeding 2 days. Such a leucocytosis does not necessarily follow splenectomy, as indicated on Charts 7 and 9. Serum protein, plasma-cell ratio, hemoglobin, and total red cells are recorded as an indirect control of changes in the blood volume.

In Rabbit 10 (Chart 10A) the latent hemopoietic response to sodium nucleinate 3 days after splenectomy occurred in 1 hour and 40 minutes, and again, 18 days after, in 2½ hours, with no tendency to leucopenia comparable with that observed under identical conditions in the normal animal. Chart 10B shows the Armeth differential count of the neutrophilic leucocytes during the first experiment with the nucleinate, the "shift to the left" being apparent within an hour and marked at 2 hours. Thus the response of the bone marrow to a chemotactic stimulus, to be discussed later, was here apparent in the total count within 2 hours and qualitatively within an even shorter period. On Chart 10A (May 5) are recorded the complete series of observations including serum protein, cell-plasma ratio, red cells, and hemoglobin taken coincidentally with the white cells



and indicating the specificity of the leucocytic response. In order that the condition existing during the leucocytosis so frequently observed on the day following the sodium nucleinate injections might be observed, an abdominal incision was made under sodium-barbital anesthesia, and total red and white counts taken simultaneously from liver, right ventricle, and ear vein. It will be seen that the red cells from the three sources showed comparable numbers and fluctuations, but that the white cells in the periphery showed fluctuations entirely independent of the liver, even in the absence of the spleen.

With the spleen eliminated from the rabbit sodium nucleinate, under the conditions outlined, repeatedly produced a leucocytosis, without preceding leucopenia, within a period of time one-half to one-sixth of that required in animals in which the spleen was intact, the latter invariably showing a profound leucopenia immediately following the nucleinate injection.

Scheunert and Krzywanek (47), following the work of Barcroft and his associates, have shown that for 1 year after splenectomy there is no change in erythrocyte volume after exercise, and they conclude that the spleen is the only organ which regulates the number of circulating erythrocytes. With reference to the white cells, Viale (48) has recently reported experiments with direct electric stimulation of the spleen of the dog exposed at laparotomy under chloral narcosis. The occurrence, immediately following faradic stimulation, of a leucopenia in the blood of the splenic vein with the differential count unchanged, and without changes comparable in direction or degree in the red cells, led him to ascribe the phenomenon to a contraction of the spleen with adherence of white cells to the vessel walls. Direct mechanical massage of the spleen gave no changes in red or white cells. He describes the spleen as an organ of mechanical regulation of the number of both red and white cells. Soler (49) found, on the basis of postsplenectomy leucocytosis, a capacity inherent in the spleen for selective regulation of the circulatory corpuscular elements. He proposes the term "sissoressi" from the Greek meaning "I accumulate." However, no critical analysis was made of the mechanism or cause of the leucocytosis he observed, and in our own experience aseptic splenectomy is not followed necessarily by a leucocytosis.

That the spleen may, under certain conditions, act as a temporary reservoir for myeloid white cells and thus exert something of a regulatory function, more or less beneficial, over their availability to the general circulation and tissues seems clear. As Krumbhaar (50) has pointed out in reviewing the anatomy of the spleen, "the shunts through the pulp offer splendid opportunities for storage and cellular metabolic mechanisms and in fact clinical evidence indicates that at times

the backwaters thus afforded may become a positive disadvantage." The leucopenia of splenic origin after sodium nucleinate represents such a "positive disadvantage." On the other hand, it is just as clear that the spleen is not the sole regulatory mechanism for white cells in the body and that its function in this direction is accessory rather than primary. In the case of the leucopenia following injections of sodium nucleinate in the normal rabbit, the spleen seems to be the only organ involved, but that this may not be true under different conditions,

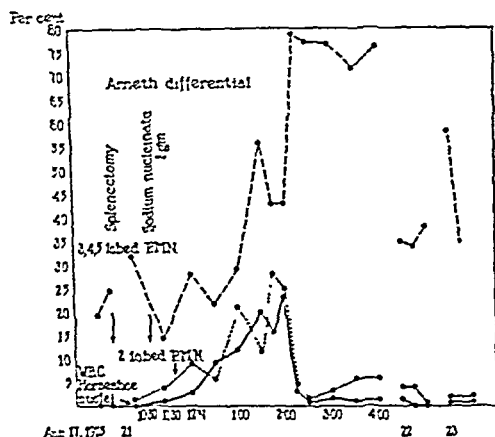


CHART 10B. Rabbit 10, T 62. In this instance the "shift to the left" in the Arneth pattern for the neutrophilic leucocytes is evident sooner than it was in the normal rabbit (see Chart 3B), and coincides with the earlier appearance of leucocytosis in the splenectomized animal. The speed of the response from the bone marrow may be deduced from the "shift" in the Arneth curve, in animals in which the spleen does not act as a reservoir for the white cells.

has been shown in the work already cited on the mechanism of leucopenia in anaphylaxis.

The Effect of Adenine and Guanine Nucleotides.

With the thought that the factor responsible for this stimulus to the neutrophilic leucocytes and apparently acting directly on the hemopoietic tissues, might reside in one of the nucleotides of which the more complex nucleinate molecule is composed, and that the cause, mechanical or chemical, of the leucopenia might be eliminated, experiments

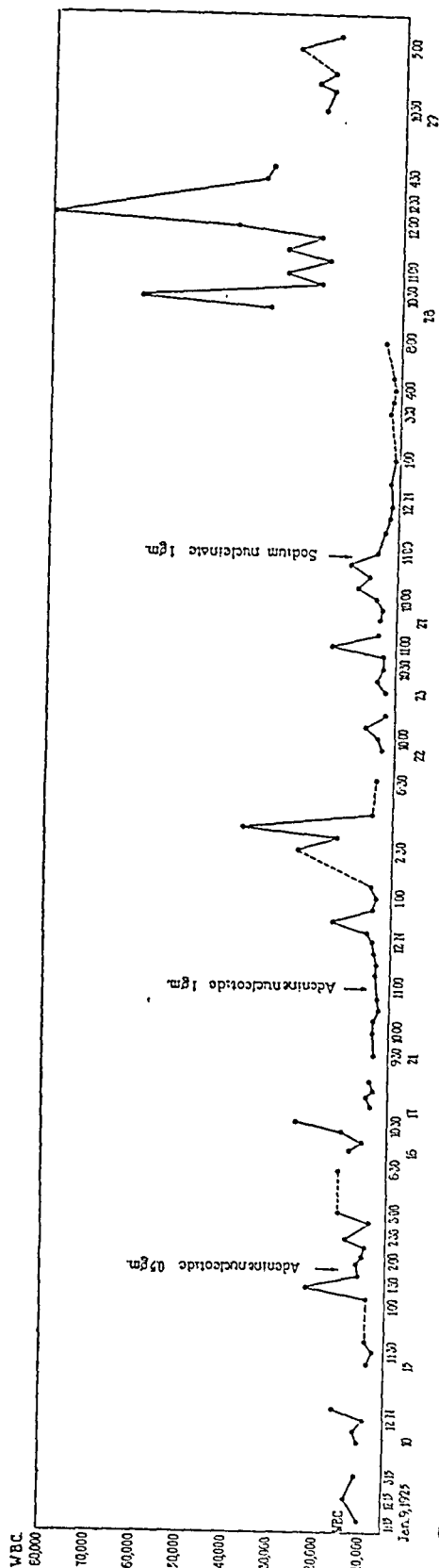


CHART 11. Rabbit 11, T 27. Adenine nucleotide (Dr. Henry Jackson, Jr.) in a dosage of 0.5 gm. was ineffective. One gm. in the same animal 6 days later produced an appreciable rise over the preliminary level within 1½ hours, and after 4 hours a count of 37,100. After another 6 days 1 gm. of sodium nucleinate induced a typical leucopenia (minimum 2900) lasting for more than 9 hours, a leucocytosis (maximum 79,500) following on the succeeding 2 days.

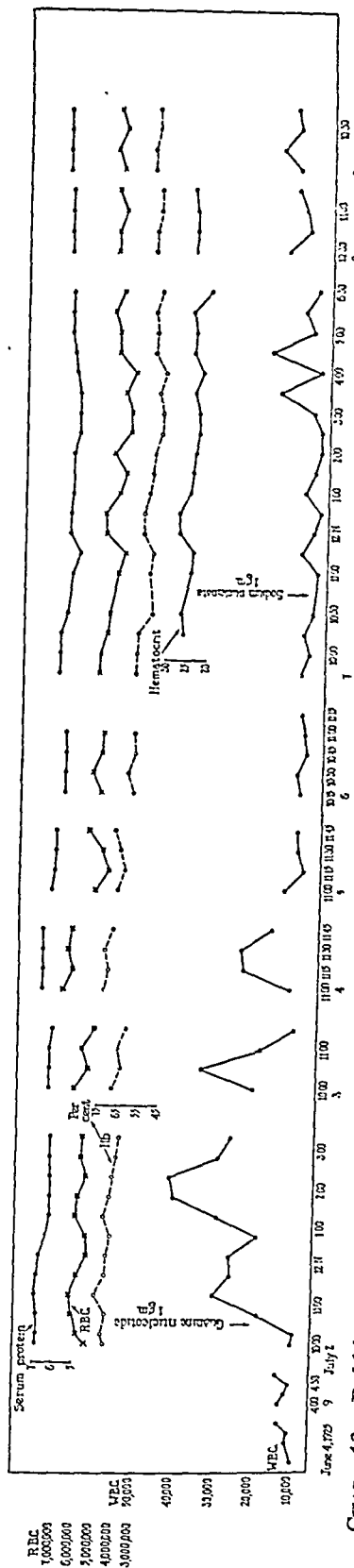


CHART 13. Rabbit 12, T 77. One gm. of guanine nucleotide produced an immediate rise in the total white count, a maximum of 41,000 being reached in 3½ hours, an increase maintained for the 2 days following. One gm. of sodium nucleinate 5 days later gave the usual leucopenia (minimum 4700), but the leucocytosis following was only moderate in degree because of slow replacement of Myelocytes C after the preceding depletion. Both the magnitude of the initial response and the rapidity of replacement of myeloid cells show variations with individual animals. Observations on the total red cells, hemoglobin, cell-plasma ratio, and serum protein show indirectly that the blood volume did not change.

were undertaken with adenine and guanine nucleotides, the only immediate split products of nucleic acid available at the moment.

Chart 11 records graphically the reactions to different dosages of adenine nucleotide and to sodium nucleinate in Rabbit 11. One-half gm. of adenine gave no detectable increase of cells on the 1st day and only a slightly suggestive response on the 2nd day. One gm. of the same substance 6 days later showed an appreciable rise above the control counts within 1½ hours of the injection, and a leucocytosis reaching 40,000 between 2 and 4 hours after. The same animal, when

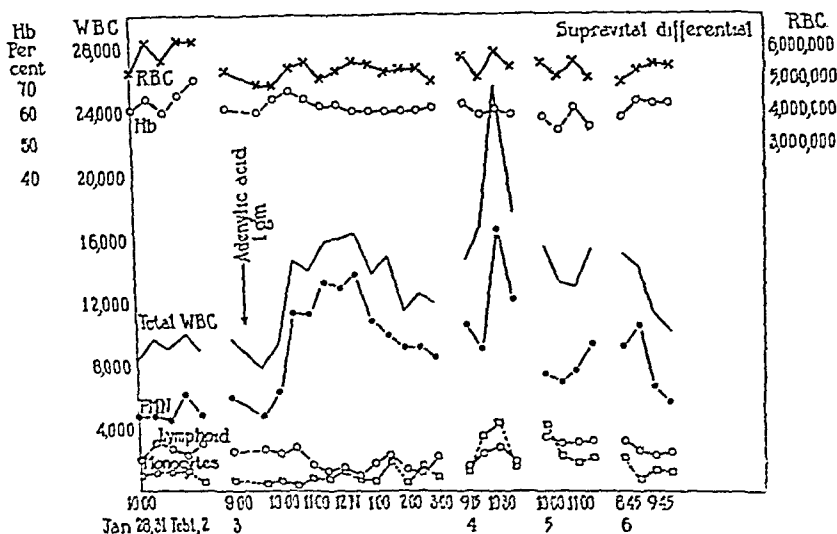


CHART 12. Rabbit 23, R 409. One gm. of adenylic acid (Dr. P. A. Levene) initiated a leucocytosis of neutrophilic origin within 1½ hours, the curves being identical in type with those produced by the guanine nucleotide in Charts 13 and 14. Differential counts of the white cells made with the supravital technic; slight lymphopenia, no lymphocytosis; monocytic rise an individual rather than a specific response probably; red cells and hemoglobin within normal limits.

given 1 gm. of sodium nucleinate 6 days after the second dose of adenine, showed a typical leucopenia lasting for more than 9 hours with a leucocytosis the 2nd day reaching 80,000.

One gm. of adenylic acid in 15 cc. of distilled water, with just enough $N/10$ NaOH to put it into solution, produced a slight increase in the neutrophilic leucocytes within 45 minutes that was well marked in 1½ hours (Rabbit 23, R 409, Chart 12). The leucocytosis was maintained during the succeeding 3 days, the highest count being recorded on the 2nd day. The differential count of the white cells made

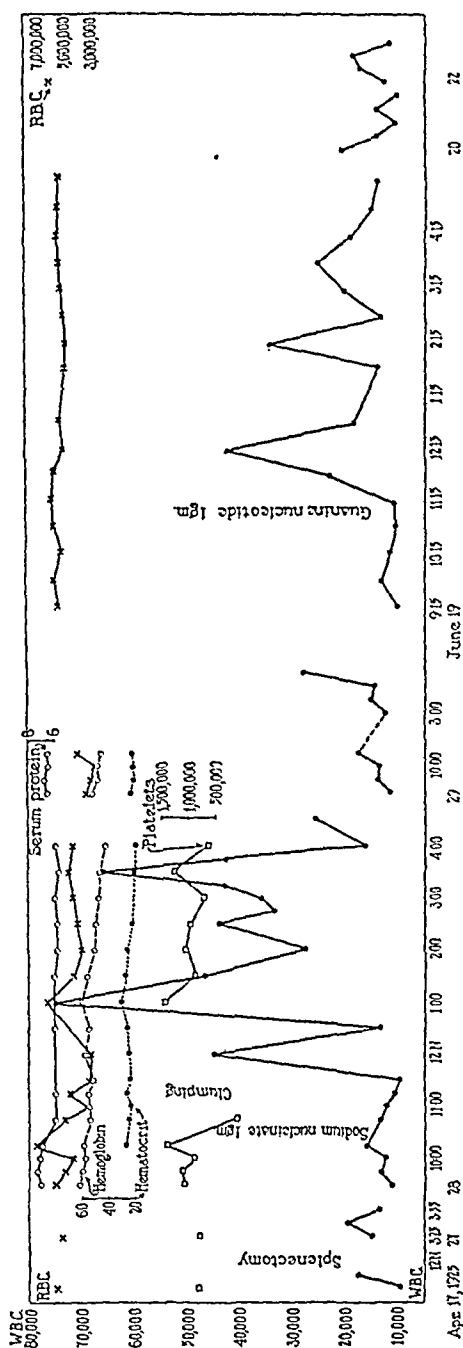


CHART 14. Rabbit 13, T 61. After splenectomy the response to sodium nucleate and guanine nucleotide gave counts of 45,300 and 42,600 respectively within 1½ hours. The nucleate gave the highest counts (maximum 75,200). Note the clumping of the platelets after sodium nucleate during the time when leucopenia is usual in non-splenectomized animals.

with the supravital technic showed the specificity of the effect on the myeloid cells with the maximum a threefold increase in polymorphonuclear neutrophils. The neutrophilic leucocytes showed unusually active motility, there was a definite increase in the percentage of younger forms, and Myelocytes C and metamyelocytes were recorded in four of the counts on the day of the injection and in one count on the 2nd day. The lymphocytes showed a slight depression on the 1st day, but no lymphocytosis followed. The monocytes in this instance were increased in total number, particularly on the 2nd and 3rd days, but this would appear to be an individual idiosyncrasy of the animal instead of a part of the usual response. Red cells and hemoglobin remained within the normal limits of variation. There was no temperature reaction.

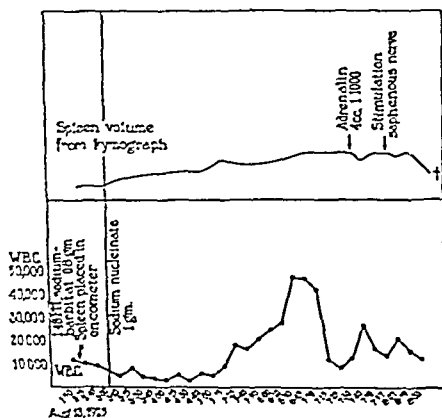


CHART 15. Rabbit 14, T 108. During the leucopenia following sodium nucleinate the spleen increases in volume. This increase is maintained through the initial period of leucocytosis, at least, and correlates with the numerical and histological evidence of greatly increased numbers of neutrophilic leucocytes in the splenic pulp. Coincident with the splenic contraction after adrenalin and after mechanical stimulation of a sensory nerve there was, in each case, a transitory increase in the white cells.

On Chart 13 may be followed the contrast in the effects of guanine nucleotide and sodium nucleinate in the same individual. The response after the administration of 1 gm. of guanine nucleotide was immediate, as evidenced by the control counts preceding. The leucocytosis reached 40,000 within 3 hours and a high count was maintained for the succeeding 2 days, the normal range being reached only on the 4th day. Five days after the guanine 1 gm. of sodium nucleinate was given, which resulted in a leucopenia lasting for more than 4 hours, the leucocytosis following being relatively low. This illustrates a latent period in the replacement of Myelocytes C after their depletion in response to the first chemotactic

substance, during which period a second chemotactic stimulus is relatively ineffective. Serum protein, red cells, hemoglobin, and cell-plasma ratio were followed in this animal, as in others, and showed no appreciable difference with the two substances. Guanylic acid in a dosage of 450 mg. (Rabbit 24, R 410) produced a proportionate immediate increase in the neutrophilic leucocytes identical with the curves shown on Charts 13 and 14 where guanine nucleotide was used. No temperature or constitutional reaction was noted.

Chart 14 shows similarity of the response to sodium nucleinate and guanine nucleotide in a splenectomized rabbit. Within $1\frac{1}{2}$ hours after sodium nucleinate the count was 45,000, while within 45 minutes after guanine nucleotide the count was 23,000, and at $1\frac{1}{2}$ hours 42,000. The highest point reached under the sodium nucleinate was 75,000, $2\frac{1}{2}$ hours after the injection, the highest point in this animal under guanine was 42,000, reached after $1\frac{1}{2}$ hours.

Both adenine and guanine nucleotides from two sources, in a dosage comparable to that used with sodium nucleinate, gave leucocytosis without preceding leucopenia, the effectiveness of the response being noted within the usual period of leucopenia after nucleinate. The leucopenia, described earlier, is, therefore, associated only with the more complex molecule, while the specificity of the stimulus to the neutrophilic leucocytes is retained by the less complex split products. In brief, the response of the splenectomized rabbit to sodium nucleinate is the same as that of the normal rabbit to the nucleotides.

The Effect of Sulfur and Peptone.

Since flowers of sulfur in olive oil given subcutaneously and peptone (Witte) given intravenously, have been used clinically to induce a leucocytosis, they were administered under controlled conditions to compare with the response to sodium nucleinate.

Fourteen mg. of flowers of sulfur in 2 cc. of olive oil were injected intramuscularly into Rabbit 20 (T 36). From a preliminary base line of 5500 to 7600, the counts rose to 10,000 and 11,000 1 hour and $1\frac{1}{2}$ hours, respectively, after the injection. These two counts were the only ones throughout the day going above 8700 and none were lower than 5500. This is well within the range of normal fluctuation for white cells in a rabbit in which counts are made every quarter hour during the day.

The injection of 1 gm. of Witte's peptone intravenously in Rabbit 21 (T 38) was followed by a fall in the white count from a preliminary zonal level of 9400-10,900 to 5200 in 7 minutes, coincident with a vasoconstriction; 15 minutes later, however, coincident with vasodilatation, the count was 10,400, and the count for the remainder of the day fluctuated between 6000 and 12,400.

The degree and the nature of the effects of nucleinate and nucleotides seem to be quite distinct from those observed in these last instances.

DISCUSSION.

The foregoing experiments reveal a prompt unequivocal response of the bone marrow, in the delivery of new mature neutrophilic leucocytes to the circulation, after the introduction intravenously of large doses of sodium nucleinate. However, there is a latent period in the replacement of the cells at the level of the Myelocyte C, in bone marrow, as shown through the inability to reproduce a comparable leucocytosis after too frequently repeated injections. Examination of the bone marrow confirms the rapid depletion of the myeloid foci followed by only a gradual replacement. Thus sodium nucleinate and adenine and guanine nucleotides contain stimuli capable of calling forth new neutrophilic cells so long as there is an adequate reserve of myeloid cells at the level of Myelocyte C, but they lack any factors specifically active for the maintenance of this level.

Ehrlich (51) defined chemotaxis as the attractive force, from microorganisms or toxins, for bringing cells from the large reserve always found in marrow. This term Cohnheim had acquired from the botanist Pfeiffer and carried over to the reaction involving the cells in inflammation (40). The first suggestion of a physiological factor with such potentialities was made by Horbaczewski (52) who correlated supposed digestion leucocytosis with an increase in the uric acid of the blood. However, Ewald (53) found uric acid relatively inactive in dogs in contrast to thymus and yeast nucleic acids and xanthine and guanine, the latter all inducing appreciable leucocytosis. Muir (54) assumed from his study of the marrow either a hyperplasia of myelocytes secondary to the exodus of leucocytes into the blood on the principle of overregeneration after repeated loss, as suggested by Weigert, or that the chemotactic substances directly stimulate cellular proliferation.

Sabin and Doan (33) have recently presented the reactions of bone marrow in terms of two factors, first a chemotactic factor (C) which acts directly on the myelocytes of Type C with an immediate response to a demand for increased leucocytes, such as that exemplified in the response to sodium nucleinate or guanine within 45 minutes to 2½ hours as here described; second, a maturation factor (M), not necessarily in association with the chemotactic factor, but which is

necessary for a maintained leucocytosis, through an active regeneration of myelocytes in the marrow without the latent period involved in Weigert's principle. Sabin (35), in a current review on bone marrow, discusses these factors in detail and presents a new basis upon which to approach the whole problem of the chemical control of the maturation and delivery of red and white cells from the hemopoietic centers. On such a basis as she formulates, it may be that nucleic acid and its immediate derivatives will be found to be important chemotactic factors used physiologically by the body in the maintenance of the normal equilibrium between supply and demand and even in pathological states. That a so called maturation factor is lacking in this group of substances would be the only conclusion justified from the data thus far accumulated. The myelocytic replacement here seems dependent only upon the principle of "overregeneration after repeated loss." Bacon, Novy, and Eppler (55) ascribe the leucocytic response in infection to non-specific stimuli arising from a quantitative alteration in the body metabolism rather than directly from infecting organisms, in other words, a response to the same stimuli which on a smaller scale produce the variations of normal values in the absence of infection. Under such a concept the importance of nucleic acid and of all the possible physiological stimuli is obvious.

SUMMARY.

The leucopenia induced by sodium nucleinate has been followed by repeated counts made simultaneously from the blood of a peripheral vein and from the internal organs, combined with a study of histological sections of the same organs taken with the counts. Measurements with the oncometer of changes in volume of the spleen have been correlated with the leucopenia and the leucocytosis following sodium nucleinate. It has thus been determined that the leucopenia is not the result of a vasomotor phenomenon, or of a change in blood volume, nor is it secondary to a retention of the white cells in the capillaries of lung or liver; it is due to the accumulation of neutrophilic leucocytes in the parenchyma of the spleen. That the spleen is solely responsible for the temporary depression of white cells in the general circulation under these conditions has been shown by splenectomy.

In splenectomized rabbits no leucopenia developed, but instead a leucocytosis due to a direct action on the bone marrow.

A profound change occurs in the distribution of the cells in the circulation at the moment of death. The liver, within a minute of the cessation of the circulation, shows a three- to fourfold increase in the number of white cells per c.mm., the differential count remaining unchanged. Thus estimations of the physiological distribution and redistribution of cells in the living state may be made only with the circulation unimpaired.

The injection into normal rabbits of adenine and guanine nucleotides, split products of nucleic acid, gave immediate leucocytosis of bone marrow origin similar to that observed with the more complex molecule when the spleen was eliminated.

The response of the bone marrow to chemotactic stimuli, such as those here used, may be reflected in the general circulation, through an absolute increase of young neutrophilic leucocytes, within a period of less than 1 hour. Within this brief period there takes place maturation from Myelocyte C and metamyelocyte into the early motile leucocyte, and the delivery of these just matured cells into the circulation. The response to one injection of nucleinate or nucleotide may persist into the 3rd and 4th days with a gradual depletion of the normal reserve of Myelocytes C in the bone marrow.

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EXPLANATION OF PLATES.

PLATE 21.

FIG. 1! Femoral bone marrow from Rabbit 6 (T 50) 1½ hours after 1 gm. of sodium nucleinate intravenously. The sinus is surrounded with neutrophilic leucocytes in active diapedesis and to right and left of the vessel may be seen cleared areas from which the myeloid elements have been withdrawn. See Chart 6. Section 10μ. Methylene blue-eosin. × about 750.

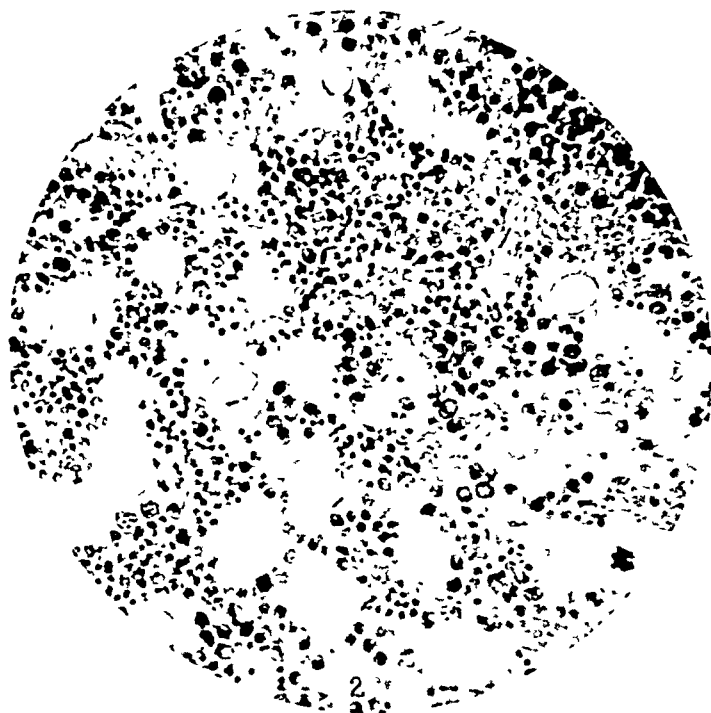
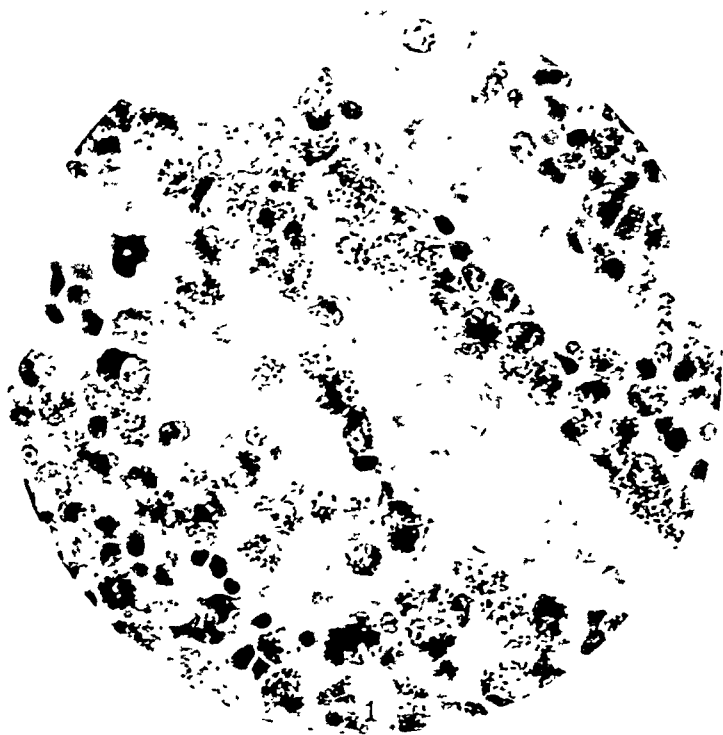
FIG. 2. Femoral bone marrow from Rabbit 22 (T 33) 18 hours after a second dose of 1 gm. of sodium nucleinate, the first having been given 4 days before. There is a decrease in size and number of fat cells in inverse proportion to the changes in the myeloid foci. It will be noted that the cells have not yet occupied all of the space made available for their development by the regression of the fat. Section 10μ. Methylene blue-eosin. × about 270.

PLATE 22.

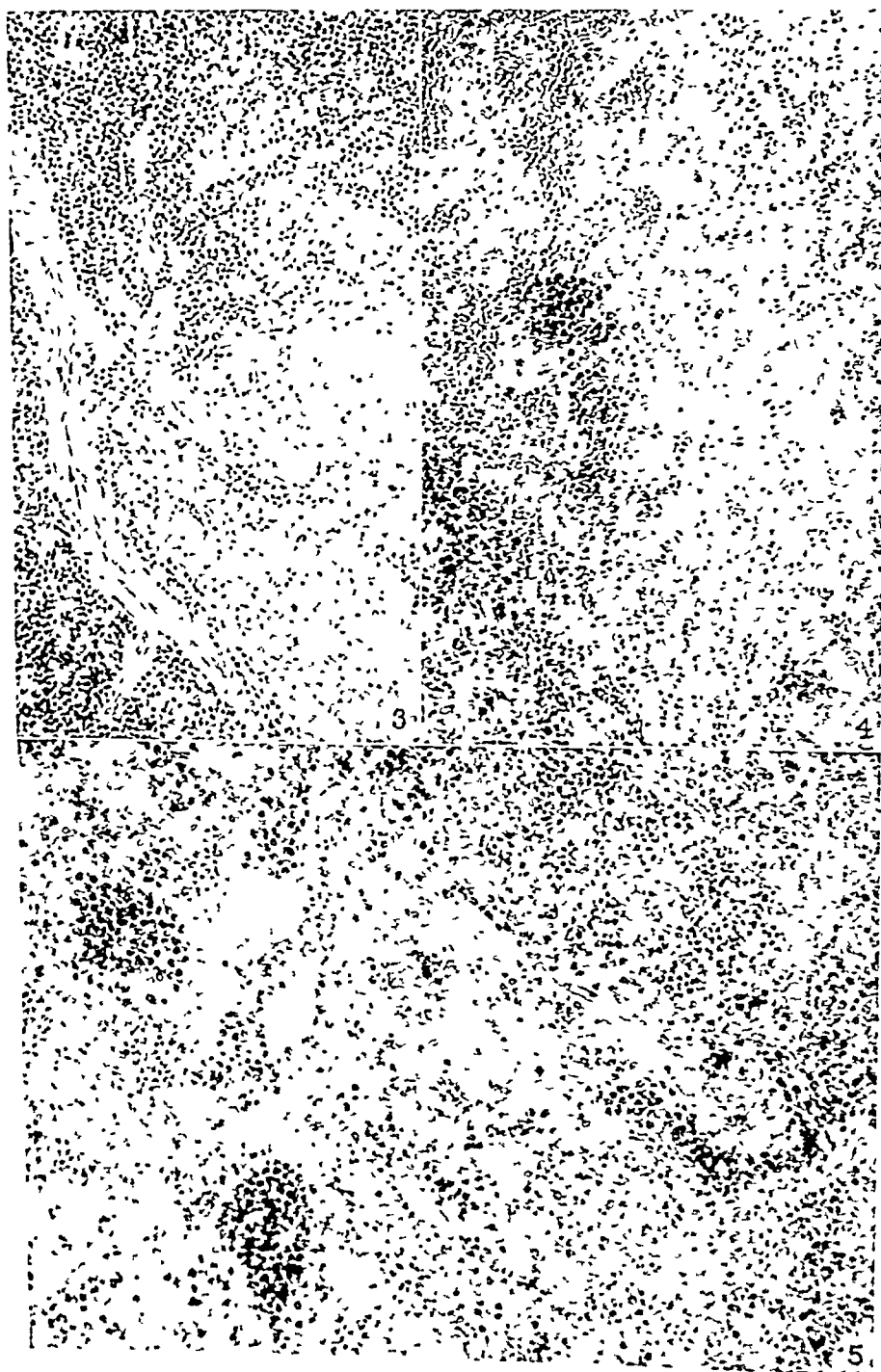
FIG. 3. Normal spleen from Rabbit 4 (T 49) 10 minutes before 1 gm. of sodium nucleinate was given intravenously. See Chart 4, Point 1. Section 10μ. Methylene blue-eosin. × 190.

FIG. 4. Sample of spleen from same experiment as Fig. 3, 1 hour after 1 gm. of sodium nucleinate intravenously. Note the beginning appearance of foci of neutrophilic leucocytes in the parenchyma. The darker cells are the neutrophilic leucocytes. The diffuse scattering of individual neutrophils throughout the pulp is not so easily recognized at this magnification. See Chart 4, Point 3. Section 10μ . Methylene blue-eosin. \times about 190.

FIG. 5. Sample of spleen from Rabbit 19 (T 31) 8 hours after 1 gm. of sodium nucleinate. Leucopenia had lasted for 5 hours, followed by 3 hours of leucocytosis. The maximum accumulation of neutrophilic leucocytes in the splenic parenchyma is still maintained. The section shows definite foci of leucocytes as well as numerous neutrophils scattered throughout the pulp. The darker cells are the neutrophilic leucocytes. 10μ . Methylene blue-eosin. \times about 190.



(Dietz, Zerkow, Warner, and Ames. Leucopenic and leucocytic states.)



(Dunn, Zerfas, Warren, and Ames. Leukopenia and its acute states.)

ALLERGIC IRRITABILITY.

IV. THE CAPACITY OF GUINEA PIGS TO PRODUCE ANTIBODIES AS AFFECTED BY THE INHERITANCE AND AS RELATED TO FAMILIAL RESISTANCE TO TUBERCULOSIS.

BY PAUL A. LEWIS, M.D., AND DOROTHY LOOMIS.

*(From the Department of Animal Pathology of The Rockefeller Institute for
Medical Research, Princeton, N. J.)*

(Received for publication, November 7, 1927.)

Wright and Lewis (1) found that certain inbred families of guinea pigs differed in their resistance to experimental tuberculosis. The work as published has been carefully repeated and considerably extended by them with particular reference to amplifying the results of cross-breeding the various families. It thus became apparent that the observed variations in familial resistance must depend on the varied inheritance of a number of factors or factor groups. The operation of at least three or four separately inherited qualities was suggested by the genetic results.* An analysis of the known physiological and structural peculiarities of the families was presented by Wright and Lewis (1) and later in great detail by Wright (2, 3), it being shown that such qualities as color, ability to grow at a certain rate, weight at a certain age, fertility as measured either by the number of young born or the percentage of young raised to the weaning age, or, finally, general vigor as estimated by these several qualities combined could be of but minor significance with relation to resistance. Thus of the total observed variation in resistance between the families about 40 per cent appeared to be dependent on characteristics which can be accounted for at present. All of the above characters combined account for but 7 per cent out of the 40 per cent mentioned leaving somewhat over 30 per cent as due to inheritance. It might

* It is expected that the amplifying data here referred to will be published in detail in the near future.

therefore, within a range of the observed variation of about 30 per cent, be hoped to discover by appropriate experiment one or more inheritable qualities which could be considered influential as determinants of resistance. The present authors set themselves this problem.

Consideration made it clear that our problem was intimately involved with the uncertainties of knowledge in regard to the natural resistance to infectious diseases in general and tuberculosis in particular. It has been our plan to fix on certain qualities, which might conceivably be influential, and to determine for each so far as possible the variability of the inbreds by family. As the result of a succession of such studies it is hoped to piece together a picture which may suggest either the inherited qualities concerned or more definitive experiments to reveal them.

Among the general biological reactions considered in this connection is that of the capacity of animals to acquire an artificial immunity or sensitization in response to treatment with antigenically active materials, that is to say their *allergic irritability* (4-6). For it must be true generally with infectious diseases that the initial stages of infection elicit immunity responses and that these influence the course of the disease, its character, and termination.

In the light of the great accumulation of knowledge with regard to specificity of immunization reactions it is possible on one view to presuppose that the quantitative relations within any such group of animals as that under consideration might vary for each antigen and that the only significant reactions for our purpose would be those connected with the tubercle bacillus or its products. But at the time this problem was taken up it was quite doubtful whether the guinea pig had ever been successfully immunized against tuberculosis, and it seemed that the effort to acquire the desired information directly would be attended with many uncertainties, if it were indeed practicable in any degree. Provisional experiments in this direction were undertaken however and may be reported upon later.

On the other hand it seemed not impossible that while immunity reactions speaking generally are so highly specific, the underlying qualities in the animal upon which they depend might be much less or not at all so; that in other words within any species an animal easy to immunize with one substance might be relatively easy to immunize

with any other, or with some others. As to this the literature afforded little information and hence experiments were undertaken with various immunity reactions which were easy to execute. We have thus studied the formation of hemolytic amboceptor for sheep and beef corpuscles, agglutinins for *Bacillus typhosus* and *Bacillus abortus* (Bang), and the reaction of anaphylaxis to sheep red blood corpuscles and to horse serum. From the beginning of the work the results have been suggestive with relation to our problem. Incidental results of considerable interest with reference to allergic irritability in general were also obtained and have been the subject of publications by Lewis and Loomis (4-6).

It was found (4, 6) that preexisting infectious disease, especially tuberculosis had a very marked stimulating influence on the quantity of antibodies produced by antigens unrelated to those involved in the disease process. This observation is pertinent to the present discussion in that it implies that a more or less common mechanism underlies the highly specific reactions of immunity with which we are more familiar and on which we are technically dependent for information in this field.

We found (5) that when the complete experiment used for the classical demonstration of the phenomena of anaphylaxis was carried through on our guinea pigs the outcome varied by families and that there was a parallelism between the reactions of the families in this experiment and their resistance to tuberculosis as determined by Wright and Lewis. Those most resistant to the disease were the least responsive in the anaphylaxis experiment.

The series of papers (particularly Lewis and Loomis (4)) also give in detail the methods which we have employed in the estimation of antibody production, some discussion of the principles underlying our problem, and a review of the pertinent literature.

Our present purpose is to present and discuss briefly such observations as we have accumulated bearing on the varying capacities of the inbred families of guinea pigs to produce antibodies, keeping in mind especially the question as to whether there is any parallel to be observed between this capacity and resistance to tuberculosis.

EXPERIMENTAL.

In the first instance groups of animals from each family were injected simultaneously with sheep red blood corpuscles and a heat-killed culture of *Bacillus typhosus*. The antibodies, anti-sheep amboceptor and anti-*typhosus* agglutinin.

TABLE I.
Order of Families Relating Antibody Production to Resistance against Tuberculosis.

T. B. resistance	Anti-sheep hemolysin		Anti-beef hemolysin		<i>B. typhosus</i> agglutinin		<i>B. abortus</i> agglutinin		<i>B. abortus</i> fixation
	1st	2nd	1st	2nd	1st	2nd	1st	2nd	
35	35 (616)	35 (3000)	35 (524)	2 (487)	13 (400)	13 (60)	35 (32)	35 (94)	35 (940)
2	13 (280)	13 (2700)	2 (343)	35 (460)	35 (93)	35 (13)	2 (17)	2 (80)	2 (658)
32	2 (182)	2 (1200)	13 (331)	13 (280)	2 (90)	2 (13)	13 (15)	13 (61)	13 (237)
13	32 (92)		32 (226)	32 (220)	32 (20)	32 (6)	32 (14)	32 (14)	32 (110)

TABLE II.

Family No.	Individual No.	Natural amboceptor undiluted serum	Acquired amboceptor—dilutions					
			1:3	1:5	1:10	1:20	1:30	1:40
35 (1/100)	1	++++*	0 +++++	0 +++++	0 +++++	0 +++++	0 +	0 0
	2	++	+++++ +++++	+++++ +++++	+++++ +++++	+++++ +++++	++ 0	0 0
	3	±	+++++ +++++	+++++ +++++	+++++ +++++	+++++ +++++	++ +	++ ++
	4	++++	0 +++++	0 ++	0 0	0 0	0 0	0 0
	5	±	0 d.	0 —	0 —	0 —	0 —	0 —
	6	0	0 +++++	0 ++	0 0	0 0	0 0	0 0
13 (1/100)	1	0	+++++ +++++	+++++ +++++	+++++ +++++	+++++ +++++	++ +	0 0
	2	++++	+++++ +++++	+++++ +++++	+++++ +++++	+++++ +++++	+++ ++	+++ 0
	3	++++	0 ++	0 0	0 0	0 0	0 0	0 0
	4	0	0 0	0 0	0 0	0 0	0 0	0 0
	5	0	0 +	0 0	0 0	0 0	0 0	0 0
	6	0	0 +	0 0	0 0	0 0	0 0	0 0
32 (1/100)	1	±	0 d.	0 —	0 —	0 —	0 —	0 —
	2	+++	+++++ d.	+++++ —	+++ —	++ —	0 —	0 —
	3	±	0 0	0 0	0 0	0 0	0 0	0 0
	4	±	++ d.	0 —	0 —	0 —	0 —	0 —
	5	++++	++ 0	0 0	0 0	0 0	0 0	0 0
	6	0	++ 0	0 0	0 0	0 0	0 0	0 0

*++++ = complete hemolysis; 0 = no hemolysis; d. = dead.

Of the two readings shown for each individual the first is for the 9th, the second for the 22nd day after injection.

TABLE II—*Concluded.*

Family No.	Individual No.	Natural amboceptor undiluted serum	Acquired amboceptor—dilutions					
			1:3	1:5	1:10	1:20	1:30	1:40
2 (1/100)	1	0	0 ++++	0 ++	0 0	0 0	0 0	0 0
	2	0	0 +++	0 +	0 0	0 0	0 0	0 0
	3	0	0 +	0 0	0 0	0 0	0 0	0 0
(1/1000)	4	++++	0 0	0 0	0 0	0 0	0 0	0 0
	5	0	0 +	0 0	0 0	0 0	0 0	0 0
			0 0	0 0	0 0	0 0	0 0	0 0
	6	+	0 +	0 0	0 0	0 0	0 0	0 0
			+	0	0	0	0	0

were determined on the 9th day after the first treatment. The same animals were then treated simultaneously with beef red blood corpuscles and a heat-killed culture of *Bacillus abortus* (Bang). Again the respective amboceptor and agglutinin titres were determined on the 9th day after injection. There were three animals in each family group. Table I in the 1st column under each subhead shows the results obtained in this experiment. The experiment was repeated using groups of ten animals for each family. The results are shown in the 2nd column under each subhead. The arrangement is by family numbers in order of decreasing titre from top to bottom. The actual average titres are shown in parenthesis and where these are considered to differ by insignificant amounts they are bracketed together. In connection with the second experiment the titres of the sera for *Bacillus abortus* in the complement fixation reaction are also shown as they emphasize the significance of the differences among sera of low agglutinin titre (last column Table I).

This type of experiment was carried through once more using beef corpuscles and the heat-killed culture of *Bacillus bovissepticus* (Strain Pn. I). Agglutinins did not develop and Family 32 was not represented. The result with reference to anti-beef hemolytic amboceptor in the terms used in the table was first, 35 (3000); second, 13 (2700); third, 2 (1200).

Several experiments were done in which much larger and repeated doses of sheep cells were given. It was thought that the higher titres of amboceptor so induced might give better definition to the place of the families. This proved not to be the case. In general either Family 13 or Family 35 were the high producers but the irregularities rather than the resemblances were emphasized in this type of ex-

periment. Family 32 was represented in but two of this group of experiments and it took a high place being highest once and second to Family 35 once.

Since the exaggeration of the antibody production seemed, as has been said, to emphasize irregularities in the relations and probably to introduce additional factors it was thought possible that the repetition of the study using the minimal amount of antigen necessary to a response might be instructive. Two experiments were conducted on this basis as follows:

In the first of these, six animals were taken from each family. They were tested individually for natural hemolysin and then injected with washed red corpuscles of the sheep in amount equivalent to 1/100 cc. and to 1/1000 cc. of whole defibrinated blood, three animals of each family being used for each dose. The animals were bled on the 9th day and on the 22nd day after injection. The titres of the serum for anti-sheep amboceptor were determined in the usual way in the presence of an excess of guinea pig complement. The results are shown in Table II.

Findings.

It was found that with reference to our problem no significance is to be attached to the presence of natural amboceptor. This is very small in amount at most and frequently undemonstrable. Each of the families shows instances of its presence in maximum and its complete absence. When present it apparently does not influence the formation of specific amboceptor as instances appear of animals with the maximum of natural amboceptor giving no increase on treatment and of those with no natural amboceptor giving a good response to the injection.

Considering the acquired antibodies by families two clearly defined groups appear. The animals of Families 35 and 13 produced much more antibody than did those belonging to Families 2 and 32. Within the groups Family 35 did somewhat better than did Family 13. No distinction of value appears between Families 32 and 2.

This experiment was repeated in its essentials with the same general result. The grouping by families appeared as before. The only difference was that within the groups on repetition Family 13 produced definitely more amboceptor than Family 35.

The animals used in the second experiment were now given a second injection of 1/100 cc. of sheep corpuscles. The results were in accord with those previously obtained when repeated doses or larger amounts of corpuscles were administered. In this response to a second injection

tion Family 13 continued to lead but the level of Family 35 was essentially equaled and actually overtopped by a small margin by Families 2 and 32.

In connection with some of the experiments reported in Table I and those when larger and repeated injections of antigen were given attention was paid to the curve of antibody production. As already reported (4) it was found that in the guinea pig the curve of anti-sheep amboceptor production in response to any injection usually presents two peaks, one at about the 9th the other at about the 22nd day. Attention has been directed to possible variants in this curve with relation to family. In the main features and especially with reference to first injections no suggestion of any difference has appeared. The maxima are found at the 9th and 22nd days for each family. There is something to suggest that with reference to the second or subsequent injections the rise to and fall from the maxima is somewhat more abrupt in the case of Family 32 as compared to the others.

Interpretation.

Classified by their capacity to produce hemolytic antibodies for sheep or beef corpuscles, or agglutinins for *Bacillus typhosus* and *Bacillus abortus* (Bang) the inbred guinea pigs we have been able to study fall into two clearly defined groups; Families 13 and 35 are high producers, low are Families 2 and 32. It is found that this grouping appears most clearly when minimal doses of antigen are used in the form of a single treatment (Table II). When the amount of antigen is somewhat increased the grouping is still presented, possibly somewhat less clearly (Table I). When the treatment is in the form of much larger single doses, or repeated injections even of those minimal in amount the groups are no longer well defined. It seems justifiable to consider that the responses to the first minimal impact with antigen most truly represent the latent capacity of the animal to react with antibody production.

In the case of injections repeated at intervals it must be true that the result is a complex made up of the initial response on the basis of latent capacity and a secondary response on the basis of a stimulated

or acquired capacity. There is no *a priori* reason why this secondary reaction should not develop on the basis of inherited characteristics as the primary one seems to do. With equal probability in anticipation it might be foreseen that the reactions of the two periods should either be closely correlated (that is dependent on the same set of characters) or quite independent.

In general if the correlation were close the results in the two periods should be of the same order and it might even be expected that those of the second period with high absolute titres of antibody should be the more distinctive. That this is not the case lends something to the view that the reaction characters underlying the two phases are at least partially distinct.

The regularity with which Family 32 takes a low place when given a single treatment of antigen in small and moderate amounts suggests that some special significance should be attached to those instances when in response to repeated treatments this family takes a high place. It may well be that the reactions peculiar to the second phase of antibody productions are also conditioned by inherited characters but somewhat different from those influential in the initial response.

It seems very likely however that the results in the second period are rendered obscure and irregular by some other influence. A scrutiny of the individual records suggests that individual differences in reaction may be relatively more pronounced in this phase. The number of animals for which individual records are available is too small to enable us to decide this point. Many of the data included in the study were based on results with serum pooled by families rather than on the average of individual tests.

The order of familial resistance to infection with the tubercle bacillus, as determined by Wright and Lewis (1), from high to low is 35, 2, 32, 13. Within this group 2 is more like 35 and 32 more closely allied to 13. Comparing the order of tuberculosis resistance with the allergic irritability, arranging the families from high to low in each case and indicating the grouping by parenthesis, gives the following:

Tuberculosis resistance (35, 2) (32, 13).
Allergic irritability (35, 13) (2, 32).

It is therefore apparent that allergic irritability as defined or recognized by the capacity to produce antibodies against the antigens here used is not perfectly correlated with and hence cannot be wholly responsible for the differences in resistance. With so much understood there are still observable relations which seem to be of interest. Thus if we were to set aside Family 13 in both cases the order of the remaining families would approximate 35, 2, 32 in each relation. This seems to be sufficient to suggest that the latent capacity to produce antibodies freely may be of some significance to the natural resistance against tuberculosis. Family 13 would in this interpretation be regarded as peculiar. In later papers we hope to be able to show that this family is also peculiar in other respects which may be significant.

In our previous paper in which anaphylaxis was used as a criterion for the allergic irritability the agreement between the results and the order of resistance was also suggestively close. If it be considered that the reaction of anaphylaxis is solely determined by the abundance of antibodies produced in response to the sensitizing injection there is developed a definite conflict between results of the two types of experiment. For whereas Family 13 which is now shown to be generally a high producer of antibodies was also found the most susceptible in the anaphylactic reaction, Family 35 which is also a high producer of antibodies was definitely the most resistant to anaphylactic shock. Two equally reasonable explanations suggest themselves. Either the two kinds of antibodies are independently produced, or there is in the anaphylactic response some other and hitherto unrecognized factor which serves to protect Family 35 in spite of its presumably high content of antibodies. This matter could be decided were it possible to make satisfactory quantitative determination of the passive transfer of anaphylaxis. This we have not so far been able to do and the nature of the differences revealed by the experiments in question remains problematical.

It may not be redundant to point out once more that in this work whenever the inbred families are found to differ significantly with respect to any quality or capacity this is to be regarded as evidence *per se* that the character is influenced by the inheritance either directly, or indirectly through other inheritable interacting characters. As

all inheritance is now held by most specialists to be Mendelian such qualities and capacities are likewise to be thought of as subject to Mendelian principles. The direct determination of the Mendelian attributes however must depend on the results obtained with intercrosses between the families. In the instance here considered, the ability to produce antibodies, we have so far been unable to trace significant differences through the reactions of the crossbreds. So that while we are able to conclude that the allergic irritability as expressed in the capacity to produce antibodies is high or low according to the inheritance we can only infer that the inheritance itself is in accord with the Mendelian conception.

SUMMARY.

The allergic irritability of closely inbred guinea pigs as represented by their capacity to produce hemolytic antibodies for beef and sheep corpuscles, and agglutinins for *Bacillus typhosus* and *Bacillus abortus* (Bang) differs by families and therefore is at least partly dependent on inherited characteristics.

These differences show an imperfect but suggestive correlation with the differences in resistance of the same families to inoculation tuberculosis as previously determined by Wright and Lewis.

The differences in antibody production also show an imperfect correlation with the differences in response in the anaphylactic reaction complex as previously determined by Lewis and Loomis.

These studies suggest very strongly that the allergic irritability is one of the several inheritable characters which form a partial basis for the natural resistance to tuberculosis.

The antibody-producing capacity is only satisfactorily defined when minimal or moderate amounts of antigen are used and this in single treatments. The irregularities in experimental result when repeated treatments or very large single treatments are used suggest that antibody production in the second or "acquired capacity" phase may rest on a somewhat different fundamental basis than the latent or potential natural capacity. There is some very slight evidence that production in the second phase may also be influenced by inherited qualities.

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ULCERATIVE TYPES AS DETERMINED BY INHERITANCE
AND AS RELATED TO NATURAL RESISTANCE
AGAINST TUBERCULOSIS: AN EXPERI-
MENTAL STUDY ON INBRED
GUINEA PIGS.

BY PAUL A. LEWIS, M.D., AND DOROTHY LOOMIS.

*(From the Henry Phipps Institute of the University of Pennsylvania, Philadelphia,
and the Department of Animal Pathology of The Rockefeller
Institute for Medical Research, Princeton, N. J.)*

PLATE 23.

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Wright and Lewis (1) found that inbred guinea pigs differed by families in their natural resistance to inoculation tuberculosis. It seemed probable to the present authors that the animal material was suitable for the determination of some at least of the physiological attributes underlying natural resistance to tuberculosis. We have previously reported studies in this general direction on the allergic irritability of these animals and of tuberculous animals in general (2-5). The present report concerns the reaction of the tissues, more particularly, but not exclusively, the skin.

The experiments are unique so far as we know both in the method of observation and the character of the animal material and there is no literature bearing directly on the work. On the other hand, the results revivify in our minds discussions of diverse questions in physiology and pathology: constitution, diathesis, predisposition and local or tissue resistance, which have been more or less continuously under observation and discussion by medical men and students of inheritance for generations. It seems proper therefore to report the experiments as directly and completely as possible and to defer reference to the observations and speculations in these various fields to later pages where the bearings of the work can be analyzed.

OBSERVATIONAL PART.

The animal material was that previously used by Wright and Lewis (1) consisting of five families of guinea pig which had been inbred by continuous brother and sister mating for a long succession of generations. The families are designated by number as 35, 2, 32, 13 and 39. They decrease in resistance to tuberculosis in the order stated.

It was thought necessary, in the course of the general experiments designed to determine resistance as measured by length of life after inoculation, to vary the method of inoculation in order to make certain that the results were independent of technical details. In due course, therefore, series of animals were inoculated directly into the skin with such amounts of virulent tubercle bacilli that death from tuberculosis might be expected in about 3 months.

The constant result of the intracutaneous inoculation is the formation of a papule which increases to a nodule of considerable size. Sooner or later the center of this nodule undergoes an ulcerative process of greater or less extent. From the time the lesion is fully developed it displays processes of healing. The ulcer shrinks and may heal over. If most of the nodule has become involved in the ulcer the whole lesion may heal and leave behind a hairless linear scar. The more usual course perhaps is that the ulcer and nodule, both reduced in size, become quiescent or stagnant and persist through the life of the animal.

When this reaction was understood it was thought that there might be some significant correlation between the development of the local lesion and the length of life of the *individual* animal. And indeed there is a relation which might be measurable between length of life and the healing process above referred to but it seems of little present significance, being apparently based on the fact that the healing process is a slow one and that only those animals which live a long time give it a chance to develop fully. The tendency to heal is exhibited by nearly if not quite all the lesions about the 3rd week. It is apparently consecutive to the discharge of matter from the nodule through the ulcer.

We have studied with care the early periods in the development of the cutaneous lesion to see whether there are discernible characteristics which might, by tendency at least, render predictable the length of life of the *individual*. Such have not been found.

When the lesions are examined with reference to the *family* there are easily recognizable qualitative differences and it has been possible to express these roughly in figures so that they acquire a certain quantitative significance. The characteristics are best developed in the case of Families 35, 13 and 39, which it will be recalled are at the extremes in resistance, 35 being the most resistant, 13 being low and 39 the lowest. The work with Families 35 and 13 as concerns this matter has been exhaustive, and warrants quite final opinions. Family 39 was available in inadequate numbers early in the observation period and more recently has been unavailable. The characteristics presented by it in certain instances were definite and easy to discriminate, but gain most of their weight in formulating conclusions from the fact that they have never appeared among the very large numbers studied from other families.

When a number of animals of the two families, 35 and 13 (we have usually used twenty of each family for an experiment), are inoculated intracutaneously at the same time and with all possible care to make the inoculation uniform as to amount and location, and when these are subjected to a daily examination, differences are definite by the 10th day and continuously so thereafter.

First recognizable is the fact that the papules and nodules are somewhat larger in the case of Family 13. The ulcerative stage also appears somewhat earlier in this family. When the lesions have reached full development at about the 20th day these general differences in size persist, and do so for as long as any considerable number of the animals remain alive with unhealed lesions.

Examined at the period of maximum size the lesions are quite different in other respects. The lesion in the case of Family 35 is firm and very sharply circumscribed on palpation. The ulcer, usually centrally situated in the lesion, is surrounded by a wide margin of firm tissue. The ulcer itself is dry, relatively shallow, is saucer shaped when round, with sharply defined margins, the whole ulcerated surface being clearly visible.

In the case of Family 13 it is frequently difficult to define the lesion by palpation. The ulcer occupies a much larger proportion of the whole lesion. The rather narrow but thick and raised margin of the ulcer is soft and edematous. The base of the ulcer is apt to be moist,

and the ulcer seems deeper because of the raised or thicker margin. The margin is excoriated and inspection does not reveal the full extent of the ulcerated area because of undermining at the edge.

The illustrations (Fig. 1, Family 35, and Fig. 2, Family 13) show these characteristics fairly in so far as they are determinable on inspection. In the plates the lesion in the case of Family 13 *appears* to be more definitely circumscribed and no larger than that pertaining to Family 35 owing to the fact that palpable qualities could not properly be considered by the artist.

It would seem possible to apply the terminology of the surgeon to these lesions and to describe them both as active, healthy ulcerations, Family 35 presenting a restrained type, Family 13 an unrestrained and tumid one. It would probably be possible in such an experiment at the period of full development to identify the family in about 75 per cent of the cases by an inspection of the lesions. The failures in identification would be almost if not quite altogether cases where Family 13 would be wrongly assigned to Family 35 on this basis.

When in one such group experiment, six animals of Family 39 were included a condition developed which is well represented in Fig. 3. During the first 20 days there was nothing to distinguish these animals from those of Family 35. Thereafter their lesions developed and maintained till death, in increasing degree, a scaly or scabby margin to the very dry ulcer. When the scabs were removed patches of excoriation were found under them and the lesion continued to spread, not as the extension of a solid nodule with a widening central ulceration, but as minute ramifying excoriating ulcerations in the more superficial layers of the skin, covered by the scaly scabs described and pictured.

In another experiment comprising twelve animals of this family but four of them developed similar lesions and in these the peculiarities were less pronounced. Animals have not been available for further tests. The significance of the result seems beyond question however for nothing approaching this lesion in type has been seen either in any of the animals of the other families or in any stock animal intracutaneously inoculated. In surgical terms this lesion could be described as an indolent excoriating ulceration.

Family 13 has presented another distinctive departure from the

other families which is of significance in the present connection. The inoculations have been practiced in or near the midline somewhat below the umbilicus. The inguinal lymph nodes on both sides are secondarily involved without exception and are usually symmetrically enlarged. In the case of this family (13) and in no other it has happened not infrequently that the inguinal lymph nodes when quite large have become adherent to the skin and this has been followed by ulceration and the later formation of a persistent discharging sinus. Moreover in many instances in this family, but in no other, smaller secondary nodules have formed along radiating lines (presumably lymphatic) between the site of the initial lesion and the inguinal nodes. These have reached a diameter of several mm. and then broken, leaving ulcers showing a considerable persistence but with a general tendency to heal.

With these types clearly in mind we have repeatedly surveyed Families 2 and 32, as well as various crossbreds having their parentage in the five family lines in all possible combinations. We have been unable to recognize any other type of lesion and *on a purely qualitative basis* we have been unable to definitely place any other family or group with reference to these types. In other words, if we had had only Families 32 and 2 and the various crossbreds, while we could have made out the types of lesion characteristic for Families 35 and 13 they would have appeared as the extremes in a variable series of individual reactions and we should not have been able to assign type to group with reference to inheritance or resistance to tuberculosis. As will be pointed out in succeeding paragraphs rough qualitative considerations suggest a definite evaluation of Families 2 and 32 also, but with the crossbreds we have been able to make no group discrimination.

In order to arrive at a roughly quantitative description of the differences we have measured the lesion in its longest direction and in the direction of a line at right angles to the longest, both in mm., and have then multiplied these together to give a value in square measure which we have termed the area. Since all the contours are rounded and the lesion is often quite round this is not a true area but an arbitrary value. The true area would be very difficult to determine in view of the irregularities in contour. The arbitrary area agrees well with the qualitative characteristics. To those accustomed to evaluate tumor forma-

tion it may appear that thickness should also be estimated and the volume be the basis for comparison. The lesion however differs from a tumor in that at the time the ulcer is established there is a discharge of matter and thereafter volume even if determinable would, we think, be less representative.

The arbitrary area of the ulcer is also determined in a similar way, and at each observation period the percentage of animals showing ulceration, and later in the experiment, complete scar formation is also noted.

The data of one such experiment in which four families were used are collected in Table I.

If the figures as given in the table for Families 13 and 35 are contrasted it is seen that for each observation period the nodules and ulcers are larger in the case of 13 than that of 35. It is also apparent that the ulcer in 13 is larger in proportion to the total area of the nodule. The percentage of animals showing ulcers is always greater for this family except during the midperiod when 100 per cent show ulcers in both. From the 68th day onward when complete healing is in evidence the number of animals showing residual scars is always much less in 13 than in 35. For these two families this is the third in a series of experiments similar in every controllable respect and with the same outcome. Family 13 then seems always to give the more severe local lesion in response to equal injections of tubercle bacilli into the skin.

With respect to Families 2 and 32 the table reveals difficulty in assigning them a definite place. Up to the 40th day they are very much alike and if the size of the ulcer alone could be considered they would in this period be clearly intermediate between 13 and 35. For the three observation periods, 19, 26 and 34 days the total lesion is even smaller than in the case of 35. The percentage of ulcers is larger than for 35. From the 40th day onward these families diverge, No. 2 rather definitely taking its place with No. 13 and No. 32 with No. 35.

The whole result as to these families would seem to place them as intermediate in respect to the character of their local lesions between Families 35 and 13, with Family 32 showing a more definite resemblance to Family 35 and Family 2 a greater likeness to Family 13.

It will be noted for all families that in the later periods of observa-

TABLE I.

Days	Lesion	Reactions by families			
		No. 35	No. 13	No. 2	No. 32
12	N	38	46	42	45
	U	3	6	4	5
	Per cent U	32	56	45	61
19	N	62	68	56	55
	U	8	13	10	10
	Per cent U	87	100	95	100
26	N	48	55	45	45
	U	6	12	9	8
	Per cent U	100	100	100	100
34	N	47	54	51	42
	U	4	11	10	9
	Per cent U	100	100	100	100
40	N	46	60	57	51
	U	3	13	11	10
	Per cent U	88	100	100	100
47	N	38	66	62	44
	U	3	14	11	8
	Per cent U	81	100	100	94
54	N	38	69	66	43
	U	3	14	11	6
	Per cent U	54	100	98	91

In the first vertical column (days) the elapsed time after inoculation is given in days. In the second vertical column there are indicated for each observation period, 1st, the average size of the nodule in sq. mm. (N); 2nd, the average size of the ulcer in sq. mm. (U); 3rd, the percentage of those animals remaining alive at any observation period which show ulceration (per cent U); and 4th, for the observation periods 68 days and succeeding, the percentage of those animals remaining alive showing only residual scars (per cent S O). Comparison of the family groups for each character observed and for each observation period is obtained by reading left to right the remaining columns headed with the family numbers 35, 13, 2, 32.

TABLE I.—*Concluded.*

Days	Lesion	Reactions by families			
		No. 35	No. 13	No. 2	No. 32
61	N	18	54	57	18
	U	3	12	13	5
	Per cent U	54	95	100	78
68	N	17	43	54	12
	U	5	9	10	4
	Per cent U	21	73	90	50
	Per cent S O	25	2	2	18
75	N	19	30	52	10
	U	4	7	11	2
	Per cent U	19	53	88	28
	Per cent S O	53	7	5	19
87	N	17	40	61	18
	U	2	6	12	4
	Per cent U	28	61	88	57
	Per cent S O	33	2	5	12
96	N	21	30	58	10
	U	4	6	12	3
	Per cent U	23	44	84	26
	Per cent S O	42	2	7	32

tion there are certain irregularities in progress from week to week in the percentages showing ulcers and scars. This is due partly to deaths, the figures being calculated on the basis of the total living at any date, and partly to the fact that progress toward healing is not steady. Ulcers heal over and break down again and again before scar formation is complete. These changes affect the average size of the ulcers but little because in this late period of successive relapses they are always very small.

It was thought possible that the different characteristics of the lesions as above described might be a reflection of an immunity developing at a different rate with advancing disease. As this if true might be associated with differences in the character of the tuberculin reaction, tuberculin skin tests were made on a selected series of these ani-

imals. A few tests were done on Family 39. Families 2 and 32 were not tested. Tests of Families 35 and 13 were adequate for conclusions.

Family 39 failed to react to tuberculin in the tests made. It seems possible that this is related to the fact that these animals are so low in resistance that the period when they could become sensitive after the establishment of the disease overlaps that period late in the disease when animals as a rule fail to react. Or it may possibly have some deeper significance.

The tests on Families 35 and 13 were interesting in that the size of the local reactive lesion in the skin differed by families in about the same degree as did the size of the initial lesion due to the living bacillus, Family 13 always presenting the larger lesion. The tests do not cover all the possible relationships as the absolute sensitivity of the animals was not measured, only the character of reaction to equivalent full doses.

The result was such as to raise a question as to whether the differences in size and quality of the skin lesions were related in any specific way to the disease-producing qualities of the tubercle bacillus. As a test in control of this point the animals were injected into the skin with various amounts of turpentine and of dilutions of tincture of cantharides, agents well known to produce acute aseptic inflammatory processes. As a reaction against these materials local nodules followed by ulcerative processes were induced, and for these also the lesions were larger and more tumid in Family 13 than in Family 35.

These processes have in common the fact that as inflammations they are characterized by a pronounced focal accumulation of leucocytes particularly the polymorphonuclear types. They were controlled by comparing mild burns of the shaved skin produced by water of various temperatures applied in flat bottom vials. The results were inconstant. No differences were made out between Families 13 and 35. The lesion here is a flat shriveling crusting disc in which the ulcerative and healing processes go on under the dead epithelium and crust without the accumulation of gross nodules.

It may therefore be concluded that the differences above described between the lesions in Families 13 and 35 are not specifically related to the injury done by the tubercle bacillus, but only incidentally to this as a part of a more general reactivity. The families differ in their

reaction to inflammatory irritants, particularly perhaps to those which cause pus formation locally, whether these act very acutely (turpentine and cantharides) or more chronically (the tubercle bacillus).

The inbred families differ from each other in another respect, of interest in the present connection, which has not so far been stated either by Wright, Wright and Lewis or the present authors. When in the course of these studies we had occasion to test the effects of the intravenous inoculation with the tubercle bacillus this was done by dissecting free the jugular vein. Families 35 and 13 were used for the comparison, twenty animals belonging to each being thus operated on in this minor way at one sitting.

In order that the experiment as a whole might have a uniform beginning we had previously practiced the technique on other animals and had standardized a simple operative procedure. It was necessary to extend the animal on a suitable board, administer an anesthetic, shave the neck, make a small incision, free and lift the vein by blunt dissection, make the injection, remove the forceps and close the wound with a single stitch. The animals weighed about 300 gm., that is they were young and the tissues were relatively soft. In case the vein or any branch of it was torn it was necessary to abandon the attempt and operate on the other side. It was found that the procedure including the change of animals on the board took 5 or 6 minutes, going on at the rate of 10 or 12 an hour. The forty animals were injected after 11 a.m. of one day. They were operated on in alternate family groups of three or four animals, and in order to finish during the working day, allowing for interruption and rest periods, it was necessary to make a conscious effort to maintain a rate of work approaching the best possible. The conditions for the following observations were therefore such that incidents which under other circumstances might have been unimpressive because of their casual and occasional character became striking by reason of the enforced attention, and were checked both by alternative repetition and the time factor involved.

It was clearly observable at once that this simple operation was easier to complete in the case of Family 35 than Family 13. In 35 the tissues were stronger, there was less loose free fat in the areolar tissue both generally in the subcutis and about the veins. The vein was thus easier to find and to expose. The vein wall was definitely stouter, less easily punctured by chance and the small branches less subject to tear. The instances in which it was necessary to go to the opposite side to complete the injection were fewer.

These differences were interpreted as evidence of some constitutional divergence in the families. The animals were more closely

scrutinized from this point of view and it was evident that making due allowance for age Family 35 is built on more compact lines and is more excitable and active. This family also has a higher muscular tonus, evident on handling. The differences in nervous irritability were, it was found, well known to the attendants. Subsequent experience has shown that these differences may be obscured by conditions. They are most apparent when the diet is somewhat restricted pointing to a general difference in "feeding quality" to use the terms of the livestock man. The differences are of the character that might generally be described as those between the "lean and hungry" type and that with the obese tendency.

These qualities as between the two families considered (35, 13) may possibly be related to the observations by Wright, that among the five families 13 was marked by the best capacity to gain weight from birth to weaning (33 days) and had the largest adult size (weight). Family 35 was second to 13 in both these respects.

But that the differences we are now considering are qualities not wholly reflected in rate of gain or adult weight as indicated by the further fact that we have so far been able to draw no definite distinction between Families 13, 2, 32 and 39, while in Wright's measurements, 2, 32 and 39 were definitely inferior to 35. It thus appears certain that the present distinctions as above described rate Family 35 as one having a definite constitutional peculiarity. Each time that we have tried to place Families 2 and 32 with respect to these qualities we have freshened an impression that 32 was more like 35 and that 2 was more like 13. This might be of interest in connection with the similar but indefinite likenesses in the character of the local ulcerative processes were it certain that the judgment in the two cases was formed independently. It is, however, true that the facts with regard to the character of the lesions were well established first and were the stimulus to the effort to formulate the impressions as to general constitution. We must therefore rest the case for general constitutional attributes on the comparative merits of the two families, 35 and 13, which even though giving an incomplete account of the animal material is essentially satisfactory in that the differences there are well defined and they are clearly at the extremes so far as these groups of animals are concerned.

SUMMARY.

In summary the observational part of this paper may be restated as follows:

1. On intracutaneous inoculation with the tubercle bacillus inbred guinea pigs develop local lesions having familial characteristics.

Family 35 which has a high natural resistance against tuberculosis as measured by length of life after inoculation develops a compact nodule with a relatively restrained type of ulceration. Family 13 which has a low natural resistance against the disease develops a softer, less well demarcated, more edematous nodule with a much less restrained, more destructive type of ulceration. Differences of the same general order develop when local lesions are created on the skin with turpentine or cantharides in these two families: In other words, the distinctions relate to the general quality of inflammatory reactions in these animals rather than to a specific reactivity against the tubercle bacillus or its product.

Families 2 and 32 which are intermediate between 13 and 35 in natural resistance develop local lesions which are on the whole intermediate also, but more like those of Family 13. In so far as it is possible to assign closer relationships it would seem that Family 32 reacts somewhat more like Family 35 and Family 2 more like Family 13.

Family 39, the lowest in natural resistance of the families available for this study presents a peculiar type of local lesion which begins with a compact nodule not to be distinguished from that of the most resistant family but often ends with an extensive indolent, excoriating, crusted ulceration. This type of lesion is not always presented by this family but has never been encountered in a very extended experience with the other families.

In the case of Family 13, but in no other, the character of the local lesion finds frequent expression in the reactions of the adjacent lymphatics and lymphatic nodes. Fresh nodules and small ulcers frequently appear along the line of the lymphatics. The lymph nodes frequently become adherent to the skin, ulcerate through and present residual persistent sinuses.

2. Families 35 and 13 present differences in the quality of their

tissues suggestive of variation in constitutional character, as observed in connection with a minor surgical operation. They also appear to differ somewhat in general conformation, muscular tone and in nervous irritability or temperament.

Interpretation.

A. Present Observations as Related to the Observed Differences in Resistance against Experimental Tuberculosis.

Our conception of disease rests on the fundamental proposition that absence of injury to either structure or function is health, and similarly when disease processes are compared, that the lesser the injury or the greater the tendency of any injury to repair the greater the approach is to health, the more favorable or inclinable to health is the condition. And in connection with local insults to tissues of all sorts age old surgical experience has crystallized into certain judgments the background for which cannot be materially reinforced by direct experiment. They are essentially axiomatic. Thus a surgeon recognizes as a healthy ulcer one which is under all the circumstances making an unmistakable active progress toward the healed condition.

It seems therefore entirely justifiable to consider that in the present experiments the observations on the local lesions exhibit a considerable, perhaps an unexpectedly considerable correlation with the previously determined general resistance of the families of animals against tuberculosis. Thus Family 39 showing by all odds the least general resistance develops a local lesion which in its later phases shows neither the tendency to heal nor any appreciable measure of the activity which experience teaches must be associated with the development of such a tendency. All of the lesions in all of the other families have shown activities which if fully developed would eventually be expected to result in a healed lesion.

Family 13, next in order of increasing resistance, and in fact all of the other families exhibit a good measure of the kind of activity usually considered essential to the establishment of a healing process. But in Family 13 this is associated most definitely with an interfering, more or less progressively destructive process which cannot at present be segregated for further description but which finds its expression in

the larger, more tumid type of lesion, and particularly in the way in which the ulcer follows out to the margin of the inflammatory area.

Family 35 exhibits an active process in which this destructive tendency is under most restraint. This family also shows the largest percentage of actually healed lesions at most times.

Families 2 and 32 exhibit active lesions on the whole less clearly defined but probably intermediate in place between 35 and 13. Based on the percentage of healed lesions eventually resulting, it appears proper to say that 32 is more like 35 and 2 more like 13.

The sum total of these relationships may be expressed in the following arrangement of family numbers in order respectively of decreasing general resistance and of decreasing reactive quality. The less definite likenesses are suggested by the brackets.

General resistance: 35, 2, 32, 13, 39.

Inflammatory reactivity: 35, 32, 2, 13, 39.

With reference to our problem of locating reactive characteristics which are both based on the inheritance and capable of accounting for differences in resistance the agreement is considerable. It seems justifiable to conclude that the variations in inflammatory character partly account for the observed differences in general resistance. The fact that Families 2 and 32 do not follow the scheme makes it clear, on the other hand, that resistance is not wholly determined by inflammatory character.

B. General Considerations with Reference to Immunity against Tuberculosis.

The immunity reactions and relationships in tuberculosis are much less well understood than is the case in many other diseases. It has been the general opinion that the humoral and cellular constituents of the blood are of relatively less significance. Much weight is laid on the tuberculin reaction and the hypersensitive state of which it is the available index. There is current a suggestion that possibly immunity in this case rests largely on some peculiar relation of the tissues to the infection and the idea is summed up in the term "tissue resistance." For the most part attempts at a better definition of this

idea experimentally have resolved themselves into a study and interpretation of one or another form of the tuberculin reaction.

Analysis of the problem of tissue resistance shows that the epithelial tissues may be left out of consideration in this instance as they are but secondarily involved in the disease. The unformed constituents of the body fluids and the polymorphonuclear series of leucocytes are not to be neglected but are outside the present argument. There remain the fixed connective tissues, the blood vessels, particularly the capillaries, and the reticulo-endothelial cell system inclusive of the phagocytic macrophages and the blood cells of the mononuclear series.

The fixed connective tissues might be thought of as the point of primary injury and they certainly appear frequently in the reparative and healing process. Lewis and Newcomer (6) attempted to show some relationship between the individual capacity of the normal rabbit to form connective tissue in response to trauma and the relative susceptibility to inoculation tuberculosis of the same animal at a slightly later period. There was no such relationship shown in the experiments. The subject is difficult of approach and the negative result of one type of experiment does not exhaust it.

The other elements involved, through the hypertrophic reactions of the blood vessels give character to the process as a granuloma and by means of the temporary organization of the other cells create the typical tubercle as a second characteristic anatomical feature of the disease. It is probably not going too far to assume that factors which determine the qualities of this imperfect and more or less transient organization of the mesothelial tissues are also likely to be factors in the resistance to the progress of tuberculosis after its establishment and less tangibly perhaps in any immunity which may be naturally possessed or acquired. In this limited sense our observations appear to be in accord with the idea that tissue resistance may be a non-specific factor of import to the natural immunity to tuberculosis. Only further study can show how far, if at all, these processes can be intrinsically modified by the acquisition of a specific immunity.

C. Genetic Considerations.

From the genetic standpoint it seems necessary to assume that we are dealing here with qualities that rest on a blending type of inherit-

ance. The reaction characters themselves merge one into the other so that it is only on the average or in extreme and therefore type cases that the distinctions can be clearly seen. Moreover, in so far as we have dealt with crossbred animals, the distinctions have been blurred and appear wholly as individual variations.

The view is held today by specialists in the field that blended inheritance is a consequence of the interaction of multiple genetic factors. This would agree with an *a priori* conception of the case since it is difficult to see how so complicated a process as an inflammatory reaction carried through from initial stages to healing could be under the control of a single or even of any very small number of functional or morphologic units. It is nevertheless possible to see in the results some suggestion of segregating unit characters. The peculiar qualities presented by the extreme cases in Family 39 are to the point. It would seem not unlikely that they rest on a recessive trait or traits. Otherwise they should occasionally be encountered in the crossbreds. Similarly it would appear that those cases in Family 13 in which the inflammation proceeds with least restraint and in which the lymphatic channels and nodes show lesions peculiar to the family, may well be evidence of action or failure to act in part controlled by a recessive character or characters since they have not been encountered in the crosses.

D. Constitutional Considerations.

Belonging essentially to the prebacteriologic era of pathology is the conception that susceptibility to infectious disease is more or less definitely related to fundamental inheritable qualities which find expression in physical conformation, that is, "physical type," and in peculiarities of function, that is "constitution." The terminology was on the whole very loose and interchangeably employed. Constitution also was often thought to be expressed in physical characteristics. When functional characteristics were thought of as directly related to disease the term "diathesis" was frequently used. Thus people of a certain inherited "constitution" were regarded as especially liable to tuberculosis, particularly to that of the lymphatic glands on the basis of a "scrofulous diathesis."

When ideas were crystallized during and after the classical bacteri-

ological studies of the latter quarter of the last century the conception of the scrofulous diathesis was first amplified in an attempt to harmonize it with new observations, and then almost if not quite discarded as being at best inadequately grounded. The considerations advanced in amplification of the conception are of considerable interest in the present connection.

It was first shown, of course, that the lymphatic lesions characteristically associated with the diathesis were tuberculous and that they had in general the same etiology as pulmonary tuberculosis. It was soon very evident that it was difficult if not impossible to discriminate between those physical characteristics which might be preexistent and possibly reflect predisposing causes, and those which were the consequences of long continued chronic disease transmitted by contact infection from generation to generation and often persisting in the individual from earliest childhood to old age. It also appeared that many of the other lesions particularly those of the skin which had frequently been regarded as evidences of a scrofulous diathesis were not tuberculous but were due to casual infection with staphylococci, streptococci and probably other microorganisms.

This recognition of many of the appearances as "consequences" greatly weakened the whole conception. The further evidence that if there was a constitutional predisposition it was not strictly specific for tuberculosis but involved other inflammatory processes as well put the question out of touch with the progressive thought of the time, which was primarily engaged in establishing specific relationships, either of etiology or immunity.

With the coincident and tremendous improvement in hygienic conditions and nutritional well being in Europe and especially in America, tuberculosis and the minor infections referred to have a greatly diminished prevalence. It is now to be accepted that practically all of the aforesaid ability to segregate a type of people having the scrofulous diathesis (if such there are) was dependent on the continued manifestation of the infections to which they are susceptible.

We think it of interest and significance that this first experimental approach to the question with suitable material develops a picture which fits so well with the conception of a scrofulous diathesis as it stood at about the beginning of the present century. We have ob-

served an inherited group of reactive qualities which are related to susceptibility to tuberculosis and also find expression in the character of the tissue changes in tuberculosis and in some simple inflammatory reactions. Respecting the limitations imposed by species differences this would seem to be as close as it could be hoped to come to an experimental definition of the "scrofulous diathesis."* It seems possible that a study of skin reactions to various inflammatory irritants, especially such as could have no antigenic properties, would develop facts of interest in connection with more general studies on human constitution.

SUMMARY.

Five families of strictly inbred guinea pig whose general resistance to experimental tuberculosis had previously been determined by Wright and Lewis have now been studied with reference to the characteristics of the local lesion produced by intracutaneous inoculation with the tubercle bacillus.

It is found that there are clearly recognizable familial types based on the size and quality of the nodular lesion, the ulcerative lesion consecutive to this and the general effectiveness of the healing process when in evidence.

Family 39 which has the lowest general resistance forms an initial papule which does not differ appreciably from that formed by Family 35 which is the most resistant. In the ulcerative stage Family 39 shows an indolent excoriating process which exhibits none of the qualities which would be expected to make for healing.

Family 35 exhibits a firm primary papule and nodule followed by an active healthy type of ulceration which is definitely restrained in comparison with that of the low resistance Family 13. Family 35 also presents the largest number of completely healed lesions in the later observation periods.

* For a modern discussion of the whole question of constitution and physical type as related to susceptibility to particular diseases or disease groups, reference may be made to the recent monograph of Draper (7) and a still more recent essay by Stockard (8). These authors do not consider the scrofulous diathesis. The most recent extended review of the subject with which we are familiar is by Schluter (9).

Family 13 shows a larger, softer, primary papule and nodule than any of the others. The ulcer when formed is less restrained; that is, it is larger in proportion to the total size of the lesion. It is also more generally destructive. This family also shows a singular tendency to the formation of secondary ulcers along the lymphatic channels leading toward the adjacent lymph nodes. The adjacent lymph nodes are likewise more severely affected and frequently ulcerate through the skin giving rise to residual discharging sinuses.

Families 2 and 32 are less definitely characterized. They are of the same order as 35 and 13 in that the ulcerations are active and healthy. Such similarities as are recognizable place Family 2 more nearly with 13 and Family 32 more nearly with 35.

In general the qualities of lesion exhibited are such as to agree with the general resistance as previously determined and it is believed that the qualities underlying the tissue reaction may be safely considered to be among the influential factors in the make-up of the natural resistance against tuberculosis.

The differences in reaction are in part manifested against agents causing simple inflammation also, and hence must be designated as non-specific with reference to the tubercle bacillus.

There are definite indications of other "constitutional" differences in the character of the tissues and the general make-up of these families.

The accumulated evidence from the study of the separate families and intercrosses between them is to the effect that the differences in question are transmitted by the blending type of inheritance and are therefore controlled by multiple unit characters. It is also suggested by the observations that certain of the significant characters are recessive in nature.

It is of considerable interest that the observations agree very well with the older conception of an inherited, predisposing, constitutional diathesis as a significant factor in the incidence of tuberculosis.

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EXPLANATION OF PLATE 23.

FIG. 1. Typical lesion on Family 35.

FIG. 2. Typical lesion on Family 13.

FIG. 3. Typical lesion on Family 39.

The animals were from a series inoculated on the same day, intracutaneously, with 1/10 mg. of a culture of bovine type of tubercle bacillus. The drawings are natural size and were made on the 20th day after inoculation.

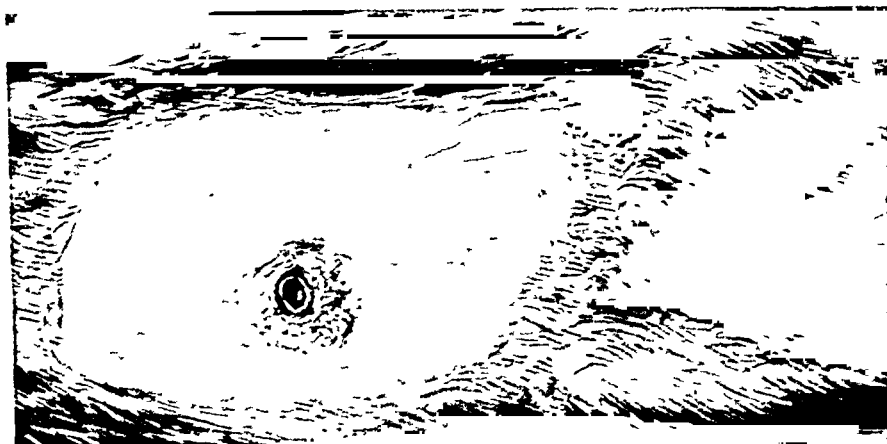


FIG. 3.



FIG. 2.



FIG. 1.

(See caption on p. 110 for descriptive types and 1st entrance.)

THE ANTIGENIC COMPLEX OF STREPTOCOCCUS HÆMOLYTICUS.

II. CHEMICAL AND IMMUNOLOGICAL PROPERTIES OF THE PROTEIN FRACTIONS.

By REBECCA C. LANCEFIELD, Ph.D.

(From the Hospital of The Rockefeller Institute for Medical Research.)

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The presence of several antigenic substances in the hemolytic streptococcus has been indicated previously; two of these were non-type-specific, while a third exhibited type specificity. Of the non-type-specific substances one was species-specific and seemed to be non-protein (1); the other, a widely reactive "nucleoprotein" fraction, was an antigenic protein and showed partial cross-reactions with similar proteins from related species of Gram-positive cocci (2). The type-specific substance was demonstrated in extracts of hemolytic streptococci by the use of sera absorbed with intact bacteria of heterologous hemolytic streptococci (3). By this means the non-type-specific antibodies were removed from the sera and the remaining type-specific antibodies were detected with the precipitin test.

This report is concerned with the chemical and immunological relationships of the type-specific substance and with a review of the properties of the group reactive nucleoprotein for purposes of comparison.

Since it was highly desirable to determine the nature of the type-specific substance, designated as M, attempts were made to isolate and purify it. With sera absorbed as described, several kinds of extracts were tested for the presence of the type-specific substance. It was found that dried bacteria pulverized in a ball mill, and extracted with $\approx/100$ NaOH in the cold, yielded some type-specific substance, M, although the non-type-specific nucleoprotein, P, predominated. Similar extraction with 0.85 per cent NaCl, instead of $\approx/100$ NaOH,

yielded a somewhat greater proportion of M than of P. However, the separation of the two substances from each other was too difficult to make either of these extracts a profitable source of M. HCl extracts, as previously described (3), yielded the largest amount of M and the smallest of P. While it was obvious that a procedure which involved heating in a boiling water bath for 15 minutes with $N/20$ HCl was a dubious method of obtaining material for chemical analysis, it was hoped that some idea of the nature of the type-specific substance might be gained and thus better methods of extraction be devised. The HCl extracts, therefore, were the main source of M. $N/20$ to $N/40$ HCl gave maximum yields of M, while $N/1$ HCl destroyed all the serologically active materials, and dilutions of $N/80$ and greater yielded only small amounts of M.

Methods.

Antibacterial sera were prepared by intravenous injections first of killed then of living cultures. The plan of immunization was a series of four daily injections followed by a rest period of 3 days. The first series consisted of four daily injections of 1 cc. of heat-killed culture, the second series consisted of 2 cc. doses, the third of 0.5 cc. doses of living culture, the fourth of 1 cc. doses of living culture the fifth of 2 cc. doses of living culture. If the precipitin titer of the test bleedings, taken 7 days after the last injection, was unsatisfactory, another series of three injections of 5 cc. each of living culture was given. This last series was repeated until a satisfactory titer was obtained, or until the animal was discarded if the immune response continued inadequate. Sometimes animals which did not yield satisfactory sera by this method were given a rest period of 6 weeks to 2 months. They were then further immunized either with one series of 2 cc. doses of killed culture followed by series of 5 cc. doses of living culture as before, or else with series of 10 cc. to 50 cc. of killed culture concentrated to 2 cc. volume.

It was usually not easy to produce antisera of sufficient potency to give satisfactory immune precipitates with the type-specific substance. Some rabbits yielded potent antisera in 6 weeks, while others of the same group of animals responded only moderately. Often it was necessary to continue immunization for several months before obtaining antisera of the high titer necessary for work with the precipitin test. Usually a high agglutinin titer was reached without difficulty. This may depend on the fact that small amounts of antibody in the serum are more readily detected by agglutination than by precipitation, while a much higher concentration of antibody is necessary if the object is the detection of small amounts of antigen. Since the latter is the case in the study of substances obtained from bacteria, a potent agglutinating serum is not sufficient but a pre-

cipitating serum of high titer must be obtained even though considerable time is required for its production.

The following was the usual method of preparing extracts of the type-specific substance. The bacteria from 18 liters of plain broth culture were suspended in 200 cc. of hot $N/20$ HCl, made by diluting $N/1$ HCl with 0.85 per cent NaCl solution, and heated for 10 minutes in a boiling water bath, then cooled and centrifuged. After removal of the supernatant fluid, a second extraction was made similarly with 200 cc. of fresh solvent. In all, six extractions were made before the bacterial residue was discarded. The volume was usually decreased to 100 cc. and then to 50 cc. for the later extractions. Preliminary experiments showed that, within reasonable limits, the yield of M was independent of the volume. The first two or three extracts contained the majority of M; but sufficient amounts were still present in the later extracts to make their preparation worth while. The supernatant fluids were combined and neutralized with $N/1$ NaOH. The precipitate which formed on neutralization was inactive serologically, hence was discarded. Crystals of sodium acetate (about 20 gm. per liter) were added to the water-clear, slightly yellowish supernatant fluid together with 3 or 4 volumes of 95 per cent alcohol. Most of the M was brought down in the resulting precipitate, while the non-type-specific C substance, probably carbohydrate in nature, was largely left in the supernatant fluid. A few reprecipitations with alcohol served to separate this material quite completely from the type-specific M. After standing in the ice box overnight, the alcoholic precipitate was removed by centrifugation and the supernatant fluid was saved for purification of the non-type-specific C. The precipitate was freed from alcohol by evaporation at 55°C . and then redissolved in 15 cc. or 20 cc. of saline; it was then centrifuged. Although most of the alcoholic precipitate was insoluble, precipitin tests showed very little less M in solution than there was in the original extract. Washings from the insoluble precipitate were added to the main solution and the insoluble part finally discarded. The M substance was reprecipitated with three volumes of 95 per cent alcohol and redissolved in a smaller volume of salt solution than before. This process was repeated five or six times. Crystals of sodium acetate were added at alternate precipitations to insure the presence of electrolytes. Only a small fraction of the second alcoholic precipitate was insoluble in salt solution; and after one or two more alcoholic precipitations all of the precipitate was readily soluble in salt solution. Further purification was effected by precipitating with a few drops of 10 per cent acetic acid. The precipitate was redissolved in salt solution by the addition of a drop of NaOH, and the process repeated several times in spite of some loss of active material. Micro-Kjeldahl determinations on the acetic acid supernatant fluids showed some purification since the removal of nitrogenous material was greater than the removal of M. After three reprecipitations with acetic acid, the precipitate was washed three times with distilled water. The test for chlorides was negative in the second and third wash waters. The precipitate was then dehydrated by washing three times with redistilled alcohol, and finally three times with

redistilled acetone. It was then filtered on hard filter paper and dried in a desiccator. The yield from 45 liters of broth culture was, in one instance, 18 mg. of a fine white powder.

The properties of the 18 mg. lot of M were as follows:¹

1. It gave a precipitate with copper sulfate.
2. It gave a violet biuret test which was not very strong.
3. With Millon's reagent it gave a white precipitate which remained on heating and did not become colored. This precipitate was not crystalline.
4. With the xanthoproteic test it gave a faint color on heating with HNO_3 which deepened slightly on the addition of NH_4OH .
5. The Molisch test was positive but not strong.
6. The percentage of total nitrogen was 14.6; while only a trace of amino nitrogen was present, too small a quantity to determine.
7. The acid equivalent was 552.

Earlier tests on less purified material showed that M withstood autoclaving for 20 minutes at 15 pounds pressure when the solution was neutral or acid ($\text{N}/20$ HCl), but not when the reaction was alkaline ($\text{N}/20$ NaOH).

Experiment 1.—Precipitin tests with the purified material were made against a number of homologous and of heterologous antibacterial sera to test its specificity. These results are recorded in Table I.

In all experiments, the final volume of precipitin tests was 0.5 cc. Each tube contained 0.1 cc. of serum. Controls of antigens alone and of sera alone were uniformly negative. The tests were read after 2 hours at 37°C . and again after standing overnight in the ice box. The overnight readings are recorded in all tables.

The extract still reacted with two homologous sera at a dilution of 1-300,000. With four heterologous sera, it was negative in all dilutions; with three heterologous sera, it gave weak reactions; and with one heterologous serum, it gave strong cross-reactions. These cross-reactions were not due to the C substance, for they were not observed with Serum Q696, known to be very potent in C antibodies; and, as will be shown later, the cross-reactions were not present after tryptic

¹ The author is greatly indebted to Drs. Heidelberger and Goebel for this analysis and for several others given in this paper, as well as for much helpful advice in the chemical procedure.

or peptic digestion of this extract. This cross-reaction was, therefore, due to a protein. Since this extract reacted very slightly, if at all, with potent anti-P sera, it seemed doubtful whether P impurities were responsible for the cross-reactions. Later work with the anaphylactic reaction definitely eliminated P as the cause of this non-type-

TABLE I.

*Precipitin Reactions.**Purified Type-Specific M Substance Prepared from HCl Extract.*

Serum			Antigen. HCl extract from Strain S43, Type S60. Final dilutions 1:		
No.	Prepared against		19,000	75,000	300,000
	Strain	Homologous type			
Q868	S60	S60	+++	++	+
Q865	"	"	+++	++	+
		Heterologous type			
Q311	S23	S23	—	—	—
Q611	"	"	+	+	±
Q612	"	"	+	0	0
Q613	"	"	—	—	—
Q244	"	"	—	±	—
Q245	"	"	—	—	—
R266	S39	"	+++*	++±	+
Q696	New York 5	Scarlatinal	—	—	—
Normal			—	—	—

*Test made at a different time: dilutions only estimates.

In all tables ±, +, ++, +++, +++++ indicate degrees of precipitation; — indicates a negative reaction; 0 indicates that test was not made.

specific reaction and necessitated the assumption of another non-type-specific protein in the antigenic complex of the hemolytic streptococcus; this fraction was present only in certain strains, and antibodies for it were only found in occasional antibacterial sera. The anaphylactic reactions which confirmed the existence of this somewhat hypothetical substance, designated as Y, are given in a subsequent paper.

In spite of the evident fact that the HCl extract was not entirely freed of non-type-specific fractions by the methods used for its purification, certain information in regard to its nature was acquired by the study of the material in this state. During the preparation and early tests it had become apparent that the type-specific substance behaved more like a protein than a carbohydrate: it was precipitated by the ordinary protein precipitants, including picric acid; and preparations, purified as described, contained 14 per cent nitrogen. The effect of removal of the NH_2 group on the serological specificity of M was, therefore, determined by treatment with nitrous acid.

TABLE II.

*Precipitin Reactions.**Effect of Removing NH_2 Group from Purified Type-Specific M Substance.*

Serum: * Q868, against Strain S60, Type S60		
Antigen: HCl extract of Strain S43, Type S60		
Final dilutions 1:	Treated with HNO_2 for 1 hr.	Blank control not treated with HNO_2
13,500	++	++++
27,000	+±	++++
54,000	+	+++
108,000	±	+±
216,000	±	+

* 0.1 cc. in each tube.

The same series of antigen dilutions tested against normal rabbit serum was completely negative.

Experiment 2.—Samples of "purified" M were treated with nitrous acid for 10 minutes and for 1 hour. After neutralization they were tested for M by means of the precipitin reaction.

Such treatment for 10 minutes caused a considerable reduction in the amount of precipitate with immune serum, and treatment for 1 hour reduced the amount very greatly, as shown in Table II. This experiment gave considerable indication that the type-specific M was a protein.

The effect of tryptic and of peptic digestion was also determined. A typical result is given in Experiment 3.

Experiment 3.—In this experiment, 2 per cent trypsin (Fairchild's) was allowed to act for 18 hours at 37°C., on concentrated M, in a solution sufficiently alkaline to give a permanent pink color with phenolphthalein. A control was included with the same amount of enzyme previously inactivated by heating in a boiling water bath for 10 minutes. Toluene was used as a preservative. After the incubation period, the digest and its control were neutralized with HCl and then heated for 10 minutes in a boiling water bath.

A similar digestion experiment was performed with pepsin in solutions made sufficiently acid with HCl to turn Congo red paper blue. After incubation, the digestion mixtures were neutralized with NaOH and heated to destroy the enzyme. Precipitin tests with these digests and with their controls are recorded in Table III.

TABLE III.

*Precipitin Reactions.**Effect of Tryptic and of Peptic Digestion on the Type-Specific M Substance.*

Serum: * Q868, against Strain S60, of homologous Type S60				
Antigen: HCl extract of Strain S43, Type S60				
Cc.**	Tryptic digest	Control with heated trypsin	Peptic digest	Control with heated pepsin
0.05	—	+++	—	+++
0.025	—	+++	—	+++
0.012	—	+++	—	+++
0.006	—	+++	—	+++
0.003	—	++	—	++
0.0015	—	++	—	++

* 0.1 cc. in each tube.

** Dilution of antigen unknown.

Table III shows that digestion of M with either pepsin or trypsin completely destroyed its ability to form precipitates with homologous antibacterial sera. Later it was determined that 0.5 per cent trypsin acting for 10 to 20 minutes was sufficient to destroy this antigen.

Seibert (4) in her studies on the chemical composition of the active principle of tuberculin, states that trypsin in neutral solution does not digest whole protein readily, although it does digest some of the split products at this pH. Following this suggestion, tryptic digestion of the M substance was carried out at pH 7.0. Digestion at this reaction was, however, just as complete as it was at an alkaline reaction.

It was also shown that the negative precipitin reactions after digestion were not due to the possible inhibiting action of digestion products since the peptic digest added to undigested M did not change the range or the degree of precipitin reactions.

The digests used in Experiment 3 were also tested against the heterologous serum which gave the best cross-precipitations. Table IV shows these tests. This non-type-specific substance also was destroyed by both tryptic and peptic digestion: the digests no longer reacted with this serum, although the controls with inactivated

TABLE IV.

Precipitin Reactions.

Effect of Tryptic and of Peptic Digestion on the Non-Type-Specific Substance in Purified HCl Extracts.

Serum: * R266, against Strain S39, of heterologous Type S23				
Antigen: HCl extract of Strain S43, Type S60				
Cc.**	Tryptic digest	Control with heated trypsin	Peptic digest	Control with heated pepsin
0.05	—	+±	0	+±
0.025	0	0	—	++±
0.012	—	+++	0	++±
0.006	0	0	—	++
0.003	—	++±	0	0

* 0.1 cc. in each tube.

** Dilution of antigen unknown. The antigen preparation in this table is the same as that used in Table III.

enzyme gave good cross-precipitations. Evidently the non-type-specific substance concerned here was a protein.

Numerous tests were made with regard to the time and the concentration of enzyme necessary for digestion of these two substances. It was observed consistently during a large series of such experiments that the non-type-specific fraction was digested much more rapidly than the type-specific. A separation of these substances on this basis was attempted, but the loss of the type-specific fraction was too great to make such a method useful.

Since the digestion experiments, supported by the nitrous acid test,

indicated clearly that the type-specific M was a protein, attempts were made to separate it from its impurities by salting out, not only from HCl extracts but also from NaOH and NaCl extracts of pulverized bacteria. Ammonium sulfate was found more satisfactory than sodium sulfate for this purpose; and a certain amount of separation of the type-specific substance from the non-type-specific could be attained. The non-type-specific substance was precipitated more readily than the type-specific by the lower concentrations of the salt; but in order to remove most of the non-type-specific materials a great loss of the type-specific substance was also sustained, although full saturation with ammonium sulfate was necessary to precipitate all of the M. This method was therefore abandoned. Purification by adsorption on voluminous alumina and on kaolin was also attempted without much success.

Finally, all available material was used in immunization and in anaphylaxis experiments, and further attempts to isolate M in a pure form were suspended until more material could be collected for this purpose. The immunization experiments follow.

Experiment 4.—Four rabbits were injected with HCl extracts from two strains of different types; two received intravenous injections of extracts from each strain. Five daily injections were given followed by a rest of 5 days. The dose was then doubled for the next five daily injections. This plan was followed for four series of injections. Test bleedings taken after the second and fourth series showed no agglutinins and no precipitins for the type-specific M or for the non-type-specific P and C substances. One rabbit from each series was then given a rest and the two remaining animals continued with all of the available material. Each received the HCl extract from 9 liters of plain broth culture in six daily injections in each of the two succeeding series of injections. At the end of this time these two animals had been in process of immunization for 10 weeks. Test bleedings after the fifth and sixth series of injections still showed no trace of antibodies. All four animals were finally given intravenous injections of whole bacteria in the same manner ordinarily employed for producing antibacterial sera. All responded promptly with the production of antibodies at the usual time after such injections. The failure to produce agglutinins or precipitins in response to the HCl extracts was not, therefore, due to inability of these rabbits to produce antibodies.

The failure to give an immune response to intravenous injections of HCl extracts could not be considered final proof that the M substance was not antigenic. The explanation might be that the rabbits had

not received enough antigen, or that the protein had been so altered by extraction with heat and hydrochloric acid that it could no longer function as an antigen. The latter hypothesis was tested as follows.

Experiment 5.—Two healthy young rabbits, weighing 1,800 gm. each, were immunized with a saline extract from Strain S43. This strain was selected because it gave a uniformly large yield of M. To the dried and pulverized bacteria 0.85 per cent NaCl solution was added in the cold; and the extract was made by shaking in the cold overnight. The smallest amount of 10 per cent acetic acid necessary to give maximum precipitation was added in the cold, and the extract again left in the ice box overnight. The precipitate, removed by centrifuging, was dissolved in a suitable small volume of saline with the aid of a few drops of N/4 NaOH. With precipitin reactions as previously described (3), the presence of M in such solutions was proved. It was similarly shown that practically all of the M had been precipitated from the original solution with the acetic acid. The object of this precipitation with acetic acid was to concentrate the M sufficiently for intravenous injections with the least harmful chemical manipulation.

Each rabbit was given such an extract from 1.5 liters of original broth culture in four daily intravenous injections. After 5 days rest, double that amount was given in three daily injections. Test bleedings 5 days later showed the presence of P antibodies but no M antibodies. One of the animals was sick and died as a result of *B. leptosepticus* infection after two more injections. The remaining animal received three more similar series of injections. The extract from 3 liters of culture was used for the third series, the extract from 19.5 liters for the fourth series, and that from 15 liters for the fifth series. In spite of the fact that this animal received the extract from a total of 42 liters of broth culture over a period of 5 weeks, and that bleedings after the fourth and fifth series of injections showed a high titer of P antibodies, still there were no traces of M antibodies. The anaphylactic test, reported in a succeeding paper, also failed to reveal the presence of M antibodies in the serum of this rabbit.

The failure to produce antibodies in these two rabbits by the injection of a simple saline extract gave weight to the previous evidence of the non-antigenicity of M which was obtained by the failure to immunize the four rabbits in Experiment 4 with HCl extract. Thus, although the type-specific substance in the hemolytic streptococcus is a protein, nevertheless, after it has been separated from the bacterial cell, it either does not give rise to antibodies when injected into rabbits intravenously, or else may do so only under conditions not yet determined. This substance seems, therefore, to be a haptene rather than a true antigen.

Solutions of the nucleoprotein P, on the contrary, are true antigens in that rabbits are easily immunized with them. Antisera from these animals are precipitated equally well by nucleoproteins from all strains of hemolytic streptococci. This has been reported previously (2) and has been repeated and confirmed in the present experiments. Nucleoproteins from non-hemolytic streptococci and from pneumococcus also precipitate these hemolytic streptococcus anti-P sera to a certain extent. This fraction of the hemolytic streptococcus is, therefore, not only not type-specific but not even species-specific. It is possible that with appropriate methods this nucleoprotein portion could be further fractionated into a strictly species-specific part and into other parts which are responsible for the cross-reactions with other species. Such methods are not at present available.

The species-specific substance first described by Hitchcock is considered in the succeeding paper, in which there is also included a discussion of all these reactive substances.

SUMMARY.

The chemical and immunological characteristics of the type-specific substance (M) of *Streptococcus hæmolyticus* are considered.

1. A summary of the evidence for the protein nature of this substance follows:

(a) It is precipitated by the usual protein precipitants such as, dilute alcohol, dilute acetic acid, and picric acid.

(b) It contains 14 per cent protein nitrogen after considerable purification.

(c) It is progressively destroyed by removal of the NH_2 group by treatment with nitrous acid.

(d) It is completely and readily digested by trypsin and by pepsin.

2. "Purified" extracts react in relatively high dilution with homologous antibacterial sera, but do not precipitate most heterologous antibacterial sera or sera potent in non-type-specific antibodies for the group reactive nucleoprotein P or for the species-specific probable carbohydrate C. Attempts to immunize rabbits with the type-specific protein have been unsuccessful, with simple salt solution extracts of

streptococci as well as with purified solutions. This protein seems, therefore, to have the characteristics of a haptene.

The type-specific substance (M) is contrasted with the so called nucleoprotein (P) which shows group relationships with nucleoproteins of related species and is the only fraction of hemolytic streptococcus extracts so far obtained which, after separation from the bacterial cell, is a true antigen leading to antibody production when injected into rabbits.

The occurrence of another non-type-specific protein (Y) is suggested by occasional cross-reactions of purified M with certain anti-bacterial sera. Since it has not been separated from extracts containing the type-specific M, little is known of it either chemically or serologically. The cross-reaction disappears on tryptic or peptic digestion of the extract. The fact that such extracts do not show cross-reactions with anti-P sera is evidence that this non-type-specific protein is not P.

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THE ANTIGENIC COMPLEX OF STREPTOCOCCUS HÆMOLYTICUS.

III. CHEMICAL AND IMMUNOLOGICAL PROPERTIES OF THE SPECIES- SPECIFIC SUBSTANCE.

By REBECCA C. LANCEFIELD, Ph.D.

(From the Hospital of The Rockefeller Institute for Medical Research.)

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In a previous paper the chemical and immunological relationships of the serologically active substances in extracts of hemolytic streptococci were discussed. The characteristics of the protein fractions, both type-specific and non-type-specific, were considered (1). The protein nature of the type-specific substance (M) was supported by its high nitrogen content (14 per cent), by its precipitation with protein precipitants, by its loss of activity on removal of the NH_2 group, and by its rapid and complete digestion with pepsin and with trypsin. Attempts to immunize rabbits with it after separation from the bacterial cell were unsuccessful. The so called nucleoprotein fraction (P) gave cross-reactions with nucleoproteins from related bacterial species and stimulated the production of antibodies in rabbits. The presence of another non-type-specific protein fraction (Y) was suggested by the cross-reactions of certain extracts consisting chiefly of the type-specific M. These cross-reactions disappeared on digestion with pepsin or with trypsin. This substance was, however, distinct from the nucleoprotein P since no cross-precipitation reactions were obtained with anti-P sera. It was not isolated but was only obtained in association with the type-specific protein.

The present paper is concerned with the species-specific substance, which Hitchcock first described as a "residue antigen" (2). This report also contains a discussion of the data concerning all these serologically active substances, including those described in the preceding paper (1).

It was desirable to collect and purify enough of the species-specific material, called the C substance, to determine its chemical nature. It exhibited an apparent chemical similarity to the specific carbohydrates of pneumococci (3), and, like these substances, it formed a typical disc precipitate with antibacterial sera; but, unlike the polysaccharides of pneumococcus and other bacteria studied from this point of view, it was not type-specific but formed precipitates with antibacterial sera made from hemolytic streptococci of all types. It was present in every kind of extract of hemolytic streptococcus. Since it was not precipitated to any extent by the addition of three or four volumes of 95 per cent alcohol or with acetic acid, the supernatant fluids of all extracts used for the preparation of the protein fractions (1) were saved for the isolation of C, which was separated as far as possible from the accompanying impurities.

Methods.

The antisera used in the following experiments were prepared as indicated in the preceding paper (1). While almost all antibacterial sera contained a moderate concentration of C antibodies, occasional sera from rabbits immunized for long periods were exceptionally potent. Intensive immunization seems required for the production of sera with a high titer of C antibodies. Hitchcock (2), who prepared numerous antisera of this sort, used very large doses of heat-killed culture in immunizing his rabbits.

The solutions which had been saved for the preparation of C were neutralized and concentrated to dryness, or to a small volume, *in vacuo* at a temperature not exceeding 37°C. After concentration enough distilled water was added to dissolve the salts; insoluble substances were discarded, and 3 volumes of 95 per cent alcohol were added to remove some of the protein. After standing overnight in the ice box, the precipitate was thrown down in the centrifuge and discarded. The supernatant fluid was again concentrated *in vacuo* and was dialyzed in a collodion sac against running water until most of the salts were removed. The C substance dialyzed through parchment membranes and also through collodion sacs which were impermeable to pneumococcus and to green streptococcus specific carbohydrates, but its passage was prevented by less permeable collodion sacs with the result that only an insignificant loss of active material was sustained in dialysis. When tests for chlorides were negative, the solution was again concentrated *in vacuo*, and sodium acetate in the proportion of 20 gm. per liter of solution together with 20 volumes of 95 per cent alcohol were added. This was left on ice overnight and the precipitate removed by centrifugation. The supernatant fluid, concentrated and tested with a potent anti-C serum, showed little C still present;

hence it was discarded. Lower concentrations of alcohol did not precipitate all of the C. The precipitate was freed of alcohol by evaporation and taken up in 16 cc. of distilled water; 0.6 cc. of 1:1 HCl was added; and a heavy precipitate, formed in the cold, was removed by centrifuging. The supernatant fluid was made strongly alkaline with NaOH and the gelatinous precipitate which formed was removed. Since precipitin tests showed that neither the acid nor the alkaline precipitates contained much C, they were discarded. A second 20 volume alcoholic precipitate was made, followed by precipitations with acid and alkali as before; the acid and alkaline precipitates were discarded. This process was repeated four or five times until the addition of acid and of alkali no longer produced a precipitate; the solution was then neutralized and 2 volumes of 95 per cent alcohol were added. A gummy, deep yellow precipitate separated out, leaving only a small proportion of coloring matter in the supernatant fluid, from which, after concentration, a second 2 volume alcoholic precipitate removed still more gummy, yellow material. The two precipitates were combined, redissolved, and reprecipitated with 2 volumes of alcohol. Since the resulting precipitate did not contain much C, it was discarded. The C was precipitated from the combined supernatant fluids by adding 20 volumes of alcohol; it was redissolved in distilled water and further purified by another removal of gummy, yellow substances by precipitation with 2 volumes of alcohol. This process was repeated until no more precipitate resulted after the fourth addition of 2 volumes of alcohol. The reaction of the solution was adjusted to pH 9, and absolute alcohol was added until a precipitate began to form. After standing overnight in the ice box, this precipitate was removed and was found to contain all of the C. After two such precipitations, the precipitate was dissolved in a small volume of distilled water, made acid by the addition of a drop of 1:1 HCl, and dialyzed against running tap water for 48 hours and then against frequent changes of distilled water for 48 hours more. The solution was then concentrated *in vacuo* and thrown into a large volume of acetone. The resulting precipitate was dried and stored in a vacuum desiccator.

Since the yield after so much manipulation was very small, the chemical data are meager. The final product was a fluffy white powder, which readily yielded a colorless solution in distilled water. The ordinary protein color tests with such solutions were negative, and the Molisch reaction was positive to the limit of the titer in the precipitin test. The optical rotation of one preparation (from Strain S23) before the final purification was -33° . After the removal of additional impurities, the optical rotation was the same. This indicated that the impurities removed were optically inactive and that the true figure for the active material was very much greater than the one obtained. Since the final yield from this lot was only 5 mg., no further determination of the optical rotation was made. The micro-Kjeldahl and the

quantitative carbohydrate determinations for this preparation were also unsatisfactory on account of the small amount of material. For another lot (from Strain S43) with a yield of 20 mg., the micro-Kjeldahl showed 4.2 per cent nitrogen and the carbohydrate determination showed on hydrolysis 28 per cent reducing sugar, calculated as glucose. Whether this large amount of nitrogen was an impurity or a part of the C substance cannot be stated without more material. It is interesting to note in this connection that the Type I pneumococcus specific substance has 4 to 5 per cent of nitrogen, apparently as an essential

TABLE I.

Precipitin Reactions.

Non-Type-Specific Reactions of the Purified C Substance, the Probable Carbohydrate.

Antigen (tested against) serum		Antigen (tested against) serum	
C from Strain S43, Type S60. Final dilutions	R446 against Strain S3, Type S3. 0.1 cc.	C from Strain S23, Type S23. Final dilutions	R446 against Strain S3, Type S3. 0.1 cc.
8,000	+±	10,000	+++±
16,000	++±	20,000	++++
32,000	+++	40,000	+++±
64,000	+++±	80,000	+++±
128,000	+++±	160,000	+++
256,000	+++	320,000	++±
512,000	++	640,000	+±
1,024,000	+	1,280,000	+
2,048,000	±	2,560,000	±
		5,120,000	—

component of the specific polysaccharide, and only 28 to 31 per cent of reducing sugars on acid hydrolysis (4). While this similarity to the figures obtained for a single small preparation of C from hemolytic streptococcus may not be significant, it is noteworthy that a specific polysaccharide of this composition is already known. These two C preparations were used in the anaphylaxis experiments to be reported in a succeeding paper and are discussed again in that connection. Their precipitin reactions are given in Table I.

Experiment 1.—A series of dilutions of each of the C substances described above was tested by means of the precipitin reaction against an antibacterial serum especially potent in C antibody, as shown in Table I.

The immune precipitates obtained with these non-type-specific C substances and antibacterial sera were always typical discs, such as have been described for the type-specific carbohydrates of other species (5). The serum used in this experiment had an especially high titer of C antibodies as shown by the good precipitates which both C preparations gave in dilutions of 1:1,000,000 with stronger reactions in higher concentrations of C. As usual with these substances, a marked prozone was present, so that still greater concentrations of C gave only negative results. The cross-reactions within the species of hemolytic streptococcus obtained by Hitchcock (2) were confirmed in the present experiment, as shown by the cross-reactions of three distinct types of hemolytic streptococci: the C substances were derived from *Types* S60 and S23, and the serum was prepared against a *Type* S3 strain. It is unnecessary to record the numerous precipitin tests performed with other C preparations and other antisera, since this experiment is typical of the non-type-specific C reactions.

The C substance did not precipitate sera prepared against the other commonly reactive fraction, the nucleoprotein P.

The effect of tryptic and of peptic digestion on the C substance was determined since these enzymes digested the type-specific M so easily. Experiment 2 was carried out for this purpose.

Experiment 2.—Fairchild's pepsin and trypsin were made up in 4 per cent solutions, the pepsin in salt solution and the trypsin in phosphate buffers. The pepsin was made sufficiently acid with HCl to turn Congo red paper blue, and the trypsin was made just alkaline to phenolphthalein with NaOH. Samples of each were heated for 10 minutes in a boiling water bath for use with the controls. A highly active C solution of undetermined concentration from Strain S23, *Type* S23, was mixed in equal parts with active and with heated pepsin and with active and with heated trypsin and incubated at 37°C. for 3 days with toluene as a preservative. The reaction was kept approximately constant throughout the experiment. Precipitin tests made daily showed that no change in titer had occurred by the end of the 3rd day; hence concentrated M extract was added in order to test the activity of the enzymes. The mixtures containing active enzyme were divided into two equal parts, and one part was heated for 10 minutes in a boiling water bath to inactivate the enzyme. Equivalent amounts of M extract from Strain S43, *Type* S60, were added to each tube, and the incubation at 37°C. continued. After 24 hours the digestion of M with trypsin was not quite complete, but digestion with pepsin was complete. The peptic digestion was therefore terminated by heating the mixtures in a boiling water bath for 10 minutes; while the tryptic

digestion was continued for another 24 hours, at which time the precipitin test showed that digestion of M was practically complete and this digestion was also terminated by inactivating the enzyme with heat. The total time of exposure of the C substance to active pepsin was 4 days, to active trypsin 5 days. The solutions were neutralized, cleared by centrifugation, and tested by setting up precipitin tests with antibacterial sera, one of which was potent in M antibodies, the other in C antibodies. Table II shows these reactions.

TABLE II.

*Precipitin Reactions.**Effect of Peptic and of Tryptic Digestion on the C Substance.*

Antigen: Digestion mixtures consisting of C from Strain S 23, [†] Type S 23, and M from Strain S 43, Type S 60				
Antigen dilutions 1:	Anti-C serum† (to test digestion of C)		Anti-M serum† (to test digestion of M)	
	Peptic digest	Undigested control with heated pepsin	Peptic digest	Undigested control with heated pepsin
2	+	+	—	+++**
4	±	±	—	++±
8	++	++	—	+++
16	++±	++±	—	++±
32	++	++	—	++
	Tryptic digest	Undigested control with heated trypsin	Tryptic digest	Undigested control with heated trypsin
2	+	+	+	+++**
10	++	++	±	+++
40	++	++	±	++
160	+	+	+	+

† Serum Q696 was against Strain New York 5, one of Dr. Dochez' scarlatinal strains, and was potent in non-type-specific C antibody.

† Serum Q868 was against Strain S60, Type S60, and was potent in M antibody for that type, but lacking C antibody.

*These were disc precipitates, characteristic of C reactions.

** These were flocculent precipitates, characteristic of M reactions.

At the end of the digestion experiment, all tubes contained mixtures of M with C, since the former was added to control the activity of the enzymes during their contact with C. All mixtures were tested against both kinds of antiserum in order to detect the presence either of M or of C. Table II shows that pepsin did not digest C during a 4 day

exposure, although the enzyme had not lost its activity, as proved by complete digestion of M added on the 3rd day of the experiment. The same kind of result is shown for the tryptic digestion experiment in the lower part of Table II. The trypsin had lost some of its activity in the course of the experiment, as indicated by the slow rate of digestion of the M added on the 3rd day and by the failure to digest this substance completely even after 48 hours contact. The greater part of the M was, however, digested; but the C was unaffected since no difference in the precipitin tests was detected in the active and the inactivated trypsin digestion mixtures. The failure of trypsin and of pepsin to digest the C substance during a prolonged exposure is convincing evidence that this substance is not an ordinary protein, although it does not eliminate the possibility that C might be digested by some other protein enzyme. However, this resistance to digestion together with the available chemical data increases the probability that the non-type-specific C substance in this species is a carbohydrate.

No extensive direct evidence is available that C in extracts is non-antigenic. No rabbits immunized with extracts for other purposes, however, have ever produced antibodies against C.

DISCUSSION.

This discussion includes a consideration of the chemical and immunological relationships of all the antigenic substances so far identified in extracts of hemolytic streptococci, and covers the data presented in both the preceding and the present papers which have shown a complicated structure of antigens in the hemolytic streptococcus. There have been demonstrated at least two non-type-specific substances in extracts of hemolytic streptococci, one protein and one probably carbohydrate, in addition to the type-specific fraction. This type-specific fraction (M), first detected by the use of antibacterial sera absorbed with heterologous hemolytic streptococci, is undoubtedly a protein. This is shown by its method of preparation, by its high nitrogen content in the form of protein nitrogen, and by the loss of activity associated with removal of the NH_2 group. In addition, the fact that it is easily digested by trypsin in alkaline or in neutral solution and by pepsin quite definitely proves its protein nature. In view of this finding, the failure to immunize rabbits with extracts containing this

substance is perplexing. The most probable explanation of this failure seems at first that in the course of extraction the protein has been denatured to such an extent that it is no longer a functional antigen, although it is highly reactive in the precipitin test, a behavior comparable to that of the type-specific carbohydrates of other species. The possibility that this result is due in the present case to denaturation has not been disproven; but the failure to immunize other rabbits with the acetic acid precipitate of simple saline extracts of pulverized bacteria offers some evidence that denaturation is not the cause of the non-antigenicity of this protein. Precipitin tests with the extract used to immunize these animals showed that the type-specific fraction (M) was present. The non-type-specific nucleoprotein (P) was also present; and antibodies were readily produced against it. Although the question of the antigenicity of the type-specific protein fraction must be left open for further work, the evidence presented suggests that it is a haptene in Landsteiner's sense (6), capable of reacting with antibodies produced in response to injection of the bacterial cell but incapable of giving rise to antibody production after separation from its cellular complex. Anaphylactic experiments, to be reported in another paper of this series, suggest the same thing. Other bacterial haptenes have been described but they are all non-protein, for example, pneumococcus and Friedländer's bacillus specific carbohydrates. Gay and Robertson (7), however, in 1913 worked with a protein, globin, which behaved immunologically in an analogous manner. Globin alone did not produce any antibody response in rabbits although it reacted with antibodies produced on injection of the combination, globin-caseinate. It is, therefore, in the same sense a haptene.

The species-specific fraction (C), first studied by Hitchcock, seems to correspond chemically and in the characteristic physical properties of its immune precipitate to the carbohydrates which in some other species are responsible for the type-specific reactions. Attempts were made to prepare sufficient quantities of this material for adequate chemical analyses, but large amounts of bacterial extract yielded such small fractions of active substance that only meager chemical data could be obtained. Such tests as could be made indicated the probability that the so called C substance was a carbohydrate. The failure of prolonged tryptic and peptic digestion to affect the precipitin

titer of this substance supported the supposition that it was not a protein, as did its passage through parchment and through certain collodion dialyzing sacs. Precipitin tests in the present experiments confirm Hitchcock's conclusion that this substance is entirely non-type-specific in its serological reactions. It is, however, unrelated to the non-type-specific nucleoprotein, since it never reacts with antisera prepared against the nucleoprotein.

The nucleoprotein antigen (P), described more fully in an earlier paper, is even less specific than the non-type-specific carbohydrate-like C substance and shows group relationships with nucleoproteins of related Gram-positive cocci, such as the non-hemolytic streptococci, the pneumococci, and the staphylococci. While such group relationships are marked, they are not absolute. Nucleoproteins within the group of the hemolytic streptococci, on the other hand, seem identical. No attempt was made to separate the nucleoprotein into a species-specific fraction and fractions reactive with other species, although it seems possible that this could be done if the proper chemical methods were available.

The nucleoprotein, P, is a true antigen and stimulates antibody production when injected into rabbits. It differs in this respect from the other reactive fractions obtained from the hemolytic streptococcus. While insufficient material was at hand for exhaustive tests in regard to the antigenicity of the C substance, considerable indirect evidence was available that it could not induce antibody formation since the serum of no rabbit immunized with extracts of hemolytic streptococci ever showed the slightest trace of reaction with purified C. The similar lack of antibody production on injection of the type-specific protein M has already been mentioned. Thus, there are probably at least two haptenes, one apparently carbohydrate and the other protein, in the antigenic complex of the hemolytic streptococcus.

The presence of still another non-type-specific protein fraction was suggested in certain of the extracts which, except for this impurity, consisted almost exclusively of the type-specific M. After purification of M from certain strains to such an extent that it gave only type-specific reactions with most antisera, it persistently gave cross-reactions with occasional heterologous antibacterial sera, unless the sera were absorbed, as previously described, with heterologous hemolytic

streptococci (1). A study of the intensities and range of these reactions together with the fact that no reaction was obtained with potent anti-P sera strongly suggests the existence of still another non-type-specific substance. Although digestion with trypsin and with pepsin shows that it is a protein, it evidently is a different protein from the non-type-specific nucleoprotein, P. Further evidence of its existence was obtained later by means of anaphylactic experiments.

The present analysis of the antigens of the hemolytic streptococcus, therefore, indicates a type-specific protein, (M), at least one non-type-specific protein (the so called nucleoprotein, P), with the possibility of another less well defined non-type-specific protein (Y), and finally a substance (C) which is probably a carbohydrate and is specific for species but not for type. The intact bacterial cell induces antibody formation against all these substances, but so far only the nucleoprotein has been found capable of eliciting antibodies after disruption of the cell body.

SUMMARY.

1. The chemical and immunological characteristics of the species-specific substance (C) of *Streptococcus hæmolyticus* are considered.

(a) It seems to be a carbohydrate because considerably purified preparations of C resisted prolonged tryptic and peptic digestion and were negative for the ordinary protein color tests but gave positive Molisch reactions to the limit of the precipitin titer. One such "purified" lot, however, had 4.2 per cent nitrogen and only 28 per cent reducing sugars on hydrolysis. Whether the nitrogen was due to impurities or was combined in the C substance itself, as is true of the Type I pneumococcus specific polysaccharide, cannot be stated without more material.

(b) The C substance forms precipitates with antibacterial sera prepared against heterologous, as well as against homologous hemolytic streptococci. These precipitates are typical discs like those formed by type-specific carbohydrates of other species of bacteria. C does not precipitate antinucleoprotein sera.

(c) While there is only slight direct evidence that the C substance is not antigenic, there is considerable indirect proof that this is the case. It probably is a haptene in the sense of Landsteiner.

2. A discussion is included of the chemical and immunological relationships of all the serologically active substances so far identified in extracts of the hemolytic streptococcus.

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W. J. GROZIER

JOHN H. NORTHROP

W. J. V. OSTERHOUT

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FURTHER OBSERVATIONS ON AN EXPERIMENTALLY PRODUCED SARCOMA OF THE CHICKEN.*

BY ERNEST STURM AND JAMES B. MURPHY, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, November 21, 1927.)

In a series of publications from The Rockefeller Institute appearing during the period from 1910 to 1914, five transplanted chicken tumors were described, all of which proved transmissible by cell-free filtrates or desiccates of the tumor material.¹ More recently several other transplantable chicken tumors have been reported and from all so far studied it has been possible to separate an agent from the cells capable of reproducing the tumors.

Murphy and Landsteiner,² in the hope of gaining some information on the nature of the causative agents of this chicken tumor group, succeeded in producing typical sarcomas by the combined injection of tar and embryonic tissue in adult hens. One of these was transplantable but all attempts to transmit it by filtrates or desiccates failed in the early generations. As this tumor remained the only transplantable chicken sarcoma which could not be transmitted by an agent separable from the cells, it was considered worth while to continue the attempts under varying conditions on the later generations. In the 3 years since the original publication, the neoplasm has been repeatedly transplanted and continues to grow quite readily. In the present report we have brought together the results of all the

* This investigation was carried on by means of funds from the Rutherford Donation.

¹ Rous, P., *J. Exp. Med.*, 1911, xiii, 397. Rous, P., Murphy, Jas. B., and Tytler, W. H., *J. Am. Med. Assn.*, 1912, lix, 1793. Rous, P., and Lange, L. B., *J. Exp. Med.*, 1913, xviii, 651. Rous, P., and Murphy, Jas. B., *J. Exp. Med.*, 1914, xix, 52. Rous, P., *J. Exp. Med.*, 1914, xix, 570. Lange, L. B., *J. Exp. Med.*, 1914, xix, 577.

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numerous attempts to disassociate an agent from the cells of this tumor, which for convenience is called Chicken Tumor 9.

Filtration Experiments.

The same general methods of filtration which gave positive results with the other chicken tumors, were used in the following experiments.

Filtration Method.—About 25 gm. of fresh tumor tissue, previously trimmed of all necrotic and muscle tissues, was finely chopped in a meat grinder and thoroughly ground in a mortar with sterile sand. To the suspension was added about 400 cc. of Ringer's solution and the entire mixture was thoroughly shaken for 20 minutes. It was then centrifuged for 15 minutes to remove the sand and solid portions of tissue, and the supernatant fluid was decanted. To it a suspension of a 24 hour culture of *B. prodigiosus* was added as a means of testing the permeability of the filter and the fluid was passed through a Berkefeld V candle. A trace of kieselguhr was added to the fresh filtrate prior to injecting. Injections of varying amounts (5 to 20 cc.) of filtrate were made into the breast muscles of normal adult chickens.

TABLE I.

Experiment No.	Size filter	Tumor generation	No. of fowls	No. of regions injected	Growths	
					+	-
1	V and N	1st A	8	16	0	16
2	V and N	1st A	10	20	0	20
3	V	2nd A	8	16	0	16
4	V	2nd C	10	20	0	20
5	V	3rd B	10	20	0	20
6	V	5th A	6	12	0	12
7	V	7th A	8	16	0	16
8	V	13th B	5	10	0	10
9	V	17th D	5	10	0	10
10	V	21st F	2	4	0	4
Msl.	V		25	48	0	48
			97	192	0	192

The above table shows the condensed results of the experiments in which Berkefeld filtrate of Chicken Tumor 9 was injected into normal adult chickens. The Msl. fowls are from later experiments in which filtrate alone was injected as control in tests when filtrate was injected with other substances.

TABLE II.

Experiment No.	Age of tumors	Size of tumor		"Takes" in tumor generation
		Right	Left	
	<i>wks.</i>	<i>cm.</i>	<i>cm.</i>	<i>per cent</i>
1	6	5.0 × 2.3	4.9 × 3.2	10
2	7	4.2 × 2.7	4.2 × 3.1	66.6
3	10	6.0 × 5.2	5.7 × 4.6	10
4	6	6.3 × 4.7		75
5	3	6.4 × 4.3	7.0 × 4.5	80
6	6	6.0 × 3.5	6.6 × 4.5	66.6
7	5	6.0 × 4.2	4.0 × 3.2	80
8	3	4.0 × 5.8	7.2 × 3.5	62.5-
9	5	6.5 × 4.5	7.0 × 4.9	50
10	7	10.0 × 5.0	10.0 × 5.0	75

The above table gives the data in regard to the tumors used in the filtration experiments summarized in Table I. It gives the size of the tumors used, the length of time since inoculation and the number of tumor "takes" in the same generation.

The filtration test in this group of ten experiments (Table I) was made with ten different tumors obtained from the first to the twenty-first generations. Ninety-seven chickens received the filtrates including those of the miscellaneous group made up of controls from other experiments. The fowls were injected generally in both breasts and kept under observation from 3 to 6 months. Not a single positive result was obtained. That this failure is not due to lack of malignancy of the tumor may be judged by the growth rate and percentage of "takes" on transplantation as shown in Table II. At the time most of the tests were made this tumor was growing at a rate quite equal to that of several of the other transplantable chicken tumors which were easily transmissible by filtrates.

The Injection of the Filtrate into Growing Embryomas.

The tumor originally developed in an embryoma and the possibility that the young elements in an actively growing tissue would create a more suitable environment for its successful transmission by filtrate led us to undertake the following experiments.

Experiment.—Hashed 7 day old chick embryos were injected into both breast muscles of five adult chickens. On the 12th day following the injection, at the time when the embryonic tissue was actively growing, 10 cc. of freshly prepared tumor filtrate was injected into and around the growing embryoma. Another group of five chickens was injected into each breast muscle with 10 cc. of a mixture composed of 12 cc. of hashed 7 day old chick embryos and 90 cc. of fresh tumor filtrate; while a control group of five chickens were injected with the Berkefeld filtrate of the tumor alone.

Several embryomas continued to grow actively for a short time after the filtrate injection but microscopic sections, prepared from pieces removed at operation, showed them to be composed of the cartilage and bone usually found in typical embryoma, without any indication of malignant transformation. These nodules eventually retrogressed and finally disappeared entirely. No evidence of tumor growth was observed in any of the fifteen chickens employed in this experiment.

Injection of the Filtrate into the Developing Embryo.

It was observed by Murphy and Rous³ that the Berkefeld filtrate of Chicken Tumor 1 rapidly gave rise to tumor nodules when the filtrate was injected into developing chick embryos. It was thought that a tumor might possibly result from the injection into the relatively unresistant chick embryo⁴ of the filtrate of Chicken Tumor 9.

Experiment.—A small rectangular piece was cut from the shell of a fertile egg by means of a shortened cataract knife. Exceptional precautions are necessary to avoid cutting through the shell membrane. With a pair of sterile forceps this membrane next was torn aside exposing the chick. A syringe of 2 cc. capacity fitted with a 1 inch, 20 gauge needle was filled with freshly prepared Berkefeld filtrate of the tumor tissue, and 1 cc. injected into the embryonic membranes. The small piece of shell was carefully replaced and the edges sealed with paraffin. The age of the embryos at the time of inoculation varied from 7 to 10 days. After inoculating, the eggs were returned to the incubator until the 19th day, when they were opened for examination. Thirty-three embryos so examined in our experiments failed to reveal any evidence of tumor nodules.

The Addition of a Mucoïd Fluid from a Filtrable Chicken Tumor.

The following experiment was planned with the possibility in mind that the mucoïd fluid, notably present in the tissue of some of the

³ Murphy, Jas. B., and Rous, P., *J. Exp. Med.*, 1912, xv, 119.

⁴ Murphy, Jas. B., *J. Exp. Med.*, 1913, xvii, 482.

filtrable chicken tumors, might have qualities of rendering the filter permeable to the causative agent of the tar tumor; for it is known that other factors beside the porosity of the filter influence the result of filtration.

Experiment.—The mucoid fluid was aspirated from a large Chicken Tumor 1 and filtered through filter paper to remove any lumps of tissue. It was then sealed in a glass tube and immersed in a water bath for 30 minutes, the temperature of which was kept at 55°C. in order to kill any tumor cells present and to render inactive the tumor-producing agent. Thirty cc. of this fluid was added to 30 gm. of finely chopped tar tumor tissue and ground with sterile sand in a mortar. The remainder of the filtering process was carried out in a manner similar to that of the previous experiments.

Six adult chickens were injected into each breast muscle with 10 cc. of this filtrate and as controls two chickens were injected with 10 cc. of the inactivated mucoid fluid. Another control group of four chickens were injected with 10 cc. of filtrate freshly prepared from the tumor tissue alone. All of the fowls remained negative for tumor growth during 2 months of observation.

Experiments with Desiccated Material from the Tar Sarcoma 9.

The failure to obtain any positive results by filtration led us to attempt a series of experiments in which desiccated Chicken Tumor 9 tissue was used in place of the filtrate. If this tumor was found resistant to drying, a partial analogy to the previously described transplantable chicken tumors could probably be established.

Experiments.—Large, actively growing tumors were removed under aseptic precautions, trimmed of all adhering muscle and necrotic tissue and ground in a meat grinder. The mashed tissue was then evenly spread over the bottom of a glass dish and placed in a desiccating jar containing a layer of sulfuric acid. The jar was evacuated to 4 mm. pressure and immediately placed in a freezing box where the temperature was several degrees below 0°C. In 3 to 4 days, or when the tissue was thoroughly dry, the scaly substance was pulverized in a mortar and about 2 gm. of this material was emulsified in 20 cc. of either sterile distilled water or Ringer's solution. From 2 to 5 cc. of this emulsion was injected into the breast muscles of normal chickens.

Five experiments were conducted with the desiccated material obtained from tumors in the first, second, third and sixth generations and in all forty-two chickens were injected into eighty-four regions. The fowls were kept under observation for from 3 to 6 months. No tumors developed. A summary is given in Table III.

TABLE III.
Summary of the Desiccation Experiments.

Experiment No.	Tumor generation	Size of tumor	Age of tumor	No. of chickens injected	No. of regions injected	Results	
		cm.				+	-
1	1st A	5.0 × 3.3 4.9 × 3.2	6	11	22	0	22
2	1st F	4.2 × 3.6 3.0 × 2.4	6	5	10	0	10
3	2nd C	4.2 × 2.6 4.2 × 3.1	7	10	20	0	20
4	3rd B	6.3 × 4.7	6	11	22	0	22
5	6th B	6.0 × 3.5 6.6 × 4.5	6	5	10	0	10
5				42	84	0	84

The Addition of Embryonic Tissue to the Desiccated Tumor Tissue.

As the original tumor was obtained by the injection of embryonic tissue and tar, as noted above, it seemed possible that the addition of some fresh, living embryonic tissue, to an emulsion of the desiccated tumor material, might produce the necessary stimulus for a positive growth.

Experiment.—A mixture was prepared consisting of equal portions of 7 day old chick embryonic tissue, and desiccated tumor tissue emulsified in Ringer's solution. Two cc. of this combination was injected into thirteen normal hens, and weekly observations were recorded for several months. As in all of the previous experiments, these animals remained negative, without suggestion of tumor formation.

Inoculation of the Developing Embryo with Desiccated Tumor Tissue.

In a series of eight experiments we injected into the chick embryo small portions of freshly prepared desiccate of tumors from the sixth to the sixteenth generations. Out of ninety-three living embryos injected between the 6th and 8th days and examined on the 18th day

of incubation, not one had developed any suggestion of tumor-like nodules.

From the results of the foregoing experiments it seems certain that Chicken Sarcoma 9 cannot be propagated from the cell-free filtrate of the tumor or the desiccated tumor tissue by any of the usual methods.

Further Attempts to Transmit Chicken Tumor 9 by the Addition of "Cultures" of This Tumor and Normal Tissues to Filtrates.

Gye⁵ has shown that it is possible to obtain growths of Chicken Tumor 1, after the filtrate of this tumor has been inactivated by chloroform, providing there is added to the filtrate an equal amount of fluid obtained from "cultures" of malignant tissue. More recently Murphy⁶ and Flu⁷ have demonstrated that not only malignant tissue but normal tissue "cultures" as well, will bring about this reactivation. With the idea that some essential factor might be removed by filtration or destroyed by drying Chicken Tumor 9, or that the agent is naturally feeble, we have attempted to supply the factor or augment the activity of this agent by the substances which activate the chloroform filtrate of Chicken Tumor 1.

Experiments.—The base of the medium used throughout these experiments was Hartley's broth to which had been added .2 per cent KCl, .7 per cent dextrose and 1 cc. of fresh rabbit serum. Pieces of tumor or embryonic tissue were introduced and the "cultures" were incubated under strict anaerobic conditions at 37.5°C.

To portions of freshly prepared Berkefeld filtrate of Chicken Tumor 9 were added equal amounts of supernatant fluid obtained from 3 day anaerobic "cultures" of rat placenta and chicken embryos. Five cc. of each of these combinations was injected into two groups of four normal hens, the experiment being controlled by injecting 10 cc. of the chicken tumor extract alone into two normal chickens. The animals were observed for a period of 2 months after which they were discarded as no tumors had developed.

In a second experiment, we mixed together equal portions of Chicken Tumor 9 filtrate and the supernatant fluid from 5 day "cultures" of the same tumor. Ten cc. of this mixture was injected into both breasts of three adult chickens. Another group of chickens were injected with 20 cc. of the extract alone to serve as controls. Not a single tumor developed from any of these injections.

⁵ Gye, W. E., *Lancet*, 1925, ii, 109.

⁶ Murphy, Jas. B., *J. Am. Med. Assn.*, 1926, lxxxvi, 1270.

⁷ Flu, P. C., *Centr. Bakt., J. Abt., Orig.*, 1926, cix, 332.

In a third experiment we attempted to activate the Chicken Tumor 9 filtrate by adding to it 7 day "cultures" of Chicken Tumor 1, but without results.

TABLE IV.

Materials	No. of fowls	No. of injections	Results	
			Positive	Negative
Filtrate alone in adult.....	97	192	0	192
Filtrate in growing embryoma.....	10	20	0	20
Filtrate in developing embryo.....	33	33	0	33
Filtrate and mucoid fluid from C. T. 1.....	6	12	0	12
Desiccate alone in adult.....	42	84	0	84
Desiccate and embryo tissue.....	13	26	0	26
Desiccate in developing embryo.....	93	93	0	93
Filtrate and "culture" fluid.....	13	13	0	13
Total.....	307	473	0	473

A summary of the various experiments with filtration and desiccation of Chicken Tumor 9 is given in Table IV. There is no indication that a substance exists separable from the cells by these methods, capable of reproducing the tumor.

Attempts to Demonstrate a Diffusible Substance from "Cultures" of Chicken Tumor 9.

While attempting to discover some method by which the hypothetical agent of the tar tumor could be separated from the cells, it was observed that the fluid from the "cultures" of this tumor sometimes produced tumors when the cultivation was made in sterile Ringer's solution and the tubes allowed to stand in the ice chest under anaerobic conditions for a period of 5 days or longer. These observations indicated the possibility that an active substance had diffused from the tumor fragments. In order to test the matter, the following experiments were planned.

Experiment.—1. To the filtrate of Chicken Tumor 9 was added an equal amount of supernatant fluid from "cultures" in Ringer's solution of Chicken Tumor 9 which had been kept in the ice chest for 5 days under strict anaerobic conditions. Two chickens were injected with 10 cc. of this mixture. One of these chickens

subsequently developed a tumor typical of Chicken Tumor 9. However, it was observed that one of the two control chickens, previously injected with 5 cc. of "cultural" fluid alone had also developed a fair sized nodule which eventually grew extensively and resembled Chicken Tumor 9.

Experiment.—2. A large number of "cultures" of Chicken Tumor 9 tissue in both Hartley's medium and Ringer's solution were prepared. The "cultures" were anaerobically sealed and placed in the ice chest for a period of 6 days. One half of each group of "cultures" were united and filtered through a Berkefeld V filter, while the fluid from the other half was decanted and centrifuged several times at high speed. Both the filtrate and the centrifuged cultures were injected into individual groups of two normal chickens each in measured amounts of 5 cc. No tumors developed in chickens injected with the filtered "cultures" from Hartley's medium, the supernatant fluid from Hartley's medium or from the injection of the filtered Ringer's solution "cultures." However, a typical Chicken Tumor 9 was observed in one of the two chickens injected with the centrifuged supernatant fluid from the Ringer's solution "cultures."

Experiment.—3. The general procedure of this experiment was identical with that of the preceding experiment. Here again it was observed that all three of the chickens injected with the filtrate were negative for tumor growth, whereas both of those injected with supernatant fluid from the Ringer's solution "cultures" developed tumors.

Experiment.—4. In this experiment we substituted sterile distilled water for Ringer's solution in one set of tubes while in another Hartley's medium was used. A long period of observation of the ten fowls used failed to show any tumors resulting from the injections.

As the filtrates of these "cultures" always failed to give tumors it was concluded that the occasional tumors resulting from the injection of the centrifuged material were due to the presence of living cells. This supposition was strengthened by the fact that the sediment contained large numbers of unquestionably living cells. The result then cannot be considered as giving evidence of the presence of an agent separable from the tumor tissue.

DISCUSSION.

The experiments reported here represent an extension of the original study of a tar tumor reported by Murphy and Landsteiner. In its general features the growth is a typical neoplasm with minor histological differences from other chicken tumors studied but it differs no more from these tumors than the individual tumors in the group differ from each other. Yet it appears to differ from all other transplant-

able chicken tumors having for their origin a spontaneous growth, in that despite many efforts no causative agent has been separated from the living cell. It is, of course, possible that some new method or change in technique may lead to a positive result, yet considering the very wide range of conditions resorted to in this study, its negative result would appear significant. The possibility that the agent might be highly susceptible to oxidation has not been completely tested but the negative results obtained in this laboratory with extracts of rat and mouse tumors filtered under anaerobic conditions indicate that this possibility is not of importance in explaining the failure in filtrability. That the agent might require contact with cells of the type from which the tumor presumably arose has been well covered by injecting the filtrate and desiccate into growing embryoma. That the failure is not due to natural resistance in the chicken is shown by the fact that the developing embryo, an organism without resistance,⁴ failed to yield growths on the injection of filtrate or desiccate.

While Chicken Tumor 9 is not so rapid in its growth as Chicken Tumor 1, yet it is more rapid than several of the other transplantable spontaneous tumors⁵ which have been easily transmitted by filtrates. It would seem, therefore, that the failure of filtrability in its case is not explainable on the basis of lack of malignancy.

For the present this tumor must stand as an exception in the chicken tumor group, in that it resembles the mammalian tumors in the failure to be transmitted by an agent separable from the living cell.

SUMMARY.

Numerous attempts have been made by us to separate from the cells of a tar sarcoma of the chicken (Chicken Tumor 9) a causative agent for the growth. Experiments with filtrates and desiccates injected as such or in combinations with embryonic tissues have all failed to give positive results. So too have injections of filtrates and desiccates into developing chick embryos failed to yield a response. The results confirm those of previous work with the tumor in this laboratory. The growth would appear to differ in a fundamental respect from all tumors of the fowl previously studied.

⁴ Tytler, W. H., *J. Exp. Med.*, 1913, xvii, 466. Rous, P., and Lange, L. B., *J. Exp. Med.*, 1913, xviii, 651. Rous, P., *J. Exp. Med.*, 1914, xix, 570.

THE USE OF HISTAMINE AS A STANDARD TEST FOR DIMINISHED RESISTANCE IN SUPRARENALECTO- MIZED RATS.

BY J. MARMORSTON-GOTTESMAN, M.D., AND J. GOTTESMAN, M.D.

(From the Division of Laboratories of Montefiore Hospital, New York.)

(Received for publication, December 8, 1927.)

The resistance of suprarenalectomized animals to a number of non-specific poisons is markedly reduced, as has been shown by several independent workers (1-6). These observers employing such poisons as cobra venom, curare, morphine, diphtheria toxin and typhoid vaccine were able to demonstrate a striking difference in resistance between normal and suprarenalectomized rats. In comparing the results obtained in different laboratories with the same poison, considerable differences in the lethal dose appeared to exist. With some of the poisons, as for example, typhoid vaccine, it seemed likely that the difference could be explained by a lack of uniformity of the preparation. Inasmuch as this test may have considerable value in future experimental work, it seemed advisable to secure additional data concerning certain of these poisons in order to find a stable substance having a relatively constant ratio between the lethal dose for suprarenalectomized rats and that for normal rats. This ratio should be at least 1:20. If such a substance could be found, the test might be standardized. In our attempts to standardize this test, we have used, up to the present time, typhoid vaccine, chloral hydrate, sodium cyanide and histamine, and the results obtained may be summarized as follows:

Typhoid Vaccine.—Marine and his coworkers (4) have shown that 75 per cent of rats are killed by 0.25 cc. of a standard typhoid vaccine within 8 days after suprarenalectomy. In a recent paper we showed (6) that suprarenalectomized rats surviving in good condition are killed by typhoid vaccine in doses of 0.4-1 cc. at the height of susceptibility (5-7 days). On repeating these experiments, it was noted

able chicken tumors having for their origin a spontaneous growth, in that despite many efforts no causative agent has been separated from the living cell. It is, of course, possible that some new method or change in technique may lead to a positive result, yet considering the very wide range of conditions resorted to in this study, its negative result would appear significant. The possibility that the agent might be highly susceptible to oxidation has not been completely tested but the negative results obtained in this laboratory with extracts of rat and mouse tumors filtered under anaerobic conditions indicate that this possibility is not of importance in explaining the failure in filtrability. That the agent might require contact with cells of the type from which the tumor presumably arose has been well covered by injecting the filtrate and desiccate into growing embryoma. That the failure is not due to natural resistance in the chicken is shown by the fact that the developing embryo, an organism without resistance,⁴ failed to yield growths on the injection of filtrate or desiccate.

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TABLE I.

Effect of Intraperitoneal Injections of Different Batches of Typhoid Vaccine on Suprarenalectomized Rats.

No. of rats	Preparation and Lot No.	Sex	Weight	Interval between operation and Injection	Amount of vaccine	Duration life after injection	No. rats killed
			gm.	days	cc.	hrs.	
3	N. Y. Board of Health 114-113	F.	165	5	1.0	12	2
		"	172	5	1.0	18	
		"	170	5	1.0	Survived.	
9		"	152	6	1.0	4½	6
		"	150	6	1.0	6	
		"	130	6	1.0	6	
		"	164	6	1.0	5	
		"	144	6	1.0	18	
		M.	192	6	1.0	Survived.	
		"	150	6	1.0	"	
		"	167	6	1.0	"	
		"	190	6	1.0	18	
4		"	180	6	1.5	10	4
		"	181	6	1.5	10½	
		"	178	6	1.5	18	
		F.	160	6	1.5	6	
2		"	132	8	1.0	8	2
		"	152	8	1.0	10	
2		M.	190	8	1.5	7	2
		"	175	8	1.5	18	
9	109-229	F.	135	8	1.0	Survived.	1
		M.	170	8	1.0	8	
		F.	154	8	1.0	Survived.	
		"	120	8	1.0	"	
		"	114	8	1.0	"	
		"	110	8	1.0	"	
		"	124	8	1.0	"	
		M.	179	8	1.0	"	
		"	190	8	1.0	"	
3	115-414	F.	153	7	1.0	"	None.
		"	165	7	1.0	"	
		M.	180	7	1.0	"	

TABLE I—*Concluded.*

No. of rats	Preparation and Lot No.	Sex	Weight	Interval between operation and injection	Amount of vaccine	Duration life after injection	No. rats killed
			<i>gm.</i>	<i>days</i>	<i>cc.</i>	<i>hrs.</i>	
6	115-414	F.	137	7	1.5	5	6
		"	172	7	1.5	18	
		"	167	7	1.5	4	
		M.	189	7	1.5	6	
		"	180	7	1.5	7½	
		F.	145	7	1.5	2	
3*	114-415	F.	157	7	0.2	8½	3
		"	185	7	0.2	18	
		"	192	7	0.2	12-18	
2	U. S. Army triple	F.	195	6	1.5	Survived.	None.
		"	180	6	1.5	"	

* See foot-note 1.

TABLE II.

The Effect of Chloral Hydrate on Normal Rats.

No. of rats	Sex	Weight	Dose per kg.	Amount injected	Results
		<i>gm.</i>	<i>mg.</i>	<i>mg.</i>	
3	F.	150	400	60	Recovered.
	"	146	400	60	"
	"	176	400	70	"
2	"	155	600	93	"
	"	150	600	90	"
3	"	155	700	113	Dead 2 hrs. 10 min.
	"	150	700	105	Recovered.
	"	168	700	117	"
3	"	157	800	125	Dead few min.
	"	155	800	125	" 30 "
	"	153	800	125	" 30 "

that different lots, even of so called standard typhoid vaccine, showed wide variations in toxicity.¹

Chloral Hydrate.—Eleven normal rats were injected subcutaneously with chloral hydrate. Of three rats injected with 800 mg. per kilo, all were killed. Of three rats injected with 700 mg. per kilo, one was killed, while all five rats injected with 400-600 mg. per kilo survived. Three suprarenalectomized rats were tested at the height of susceptibility (6 days after the removal of the glands), but no decrease in resistance could be demonstrated. See Tables II and III.

Sodium Cyanide.—The subcutaneous M.L.D. of sodium cyanide for normal rats was found to be between 9.8 and 11.0 mg. per kilo, approximately the same dose as that found by Voegtlin, Johnson and

TABLE III.
The Effect of Chloral Hydrate on Suprarenalectomized Rats.

No. of rats	Sex	Weight	Dose per kg.	Amount injected	Interval between operation and injection	Results
		gm.	mg.	mg.	days	
3	F.	105	400	42	9	Recovered.
	M.	210	400	84	9	"
	"	210	400	84	9	"

Dyer (7). This amount injected subcutaneously killed five out of six normal rats. Amounts less than 9.8 mg. per kilo did not kill any in a series of six normal rats used. Thirteen suprarenalectomized rats were injected subcutaneously with varying amounts of sodium cyanide. Two rats injected 6 days after removal of the glands

¹ Preparation 114, Lot 415 New York Board of Health vaccine killed with a dose of 0.2 cc. This particular vaccine had been used as a non-specific therapeutic measure in chronic arthritis and cancer, and the clinicians had noted much more marked reactions from a given dose of this preparation than had been obtained with the same dose of other preparations. The variations in toxicity which we had found would explain the differences in results reported by several workers and make typhoid vaccine unsatisfactory as a routine test substance for the resistance of suprarenalectomized rats unless the lethal dose of each lot is separately determined.

TABLE IV.
Effect of Sodium Cyanide on Normal Rats.

No. of rats	Sex	Weight	Dose per kg.	Amount injected	Results
		gm.	mg.	mg.	
3	F.	147	5.5	.80	Recovered.
	M.	164	5.5	.90	"
	F.	138	5.5	.76	"
3	"	144	4.4	.64	"
	M.	184	4.4	.80	"
	"	176	4.4	.78	"
3	F.	136	9.8	1.33	Dead 5 hrs.
	M.	144	9.8	1.40	" 2 "
	"	179	9.8	1.76	" 2 "
3	F.	144	11.0	1.58	Recovered.
	M.	184	11.0	2.01	Dead 1 hr.
	"	176	11.0	1.93	" 1 "

TABLE V.
The Effect of Sodium Cyanide on Suprarenalectomized Rats.

No. of rats	Sex	Weight	Dose per kg.	Amount injected	Interval between operation and injection	Results
		gm.	mg.	mg.	days	
2	F.	150	5.5	.82	6	Killed 3½ hrs.
	M.	180	5.5	.99	6	" 7½ "
2	"	215	2.75	.59	6	Recovered.
	F.	155	2.75	.43	6	"
6	M.	184	4.8	.9	13	Killed 1 hr.
	F.	129	4.8	.63	13	" 18 hrs.
	"	107	4.8	.52	13	" 18 "
	"	132	5.5	.73	13	Recovered.
	M.	150	5.5	.82	13	Killed 1 hr.
	"	166	5.5	.91	13	" few min.
3	"	164	4.85	.8	30	Recovered.
	"	172	4.65	.84	30	"
	F.	121	3.93	.49	30	"

with 5.5 mg. of NaCN per kilo were killed. Two rats injected at 6 days with 2.75 mg. per kilo were not killed. Five out of six rats injected 13 days after suprarenalectomy were killed with 4.8-

TABLE VI, *a*.

Effect of Intravenous Injections of Histamine Acid Phosphate on Normal Rats.

Rat No.	Weight	Histamine acid phosphate per kg.	Remarks
	gm.	mg.	
158-2	195	900	Recovered in 3 hrs. In good condition after 2 wks.
158-1	200	1000	Recovered in 3 hrs. In good condition after 2 wks.
158-4	170	1200	Recovered in good condition in 3 hrs. Lived for 6 wks. Died.
169-3	260	1350	Recovered in 3 hrs. In good condition after 2 wks.
158-3	180	1500	Recovered in good condition after 3 hrs. In good condition after 2 wks.
159-2	165	2000	Dead in 2 hrs. 15 min.

TABLE VI, *b*.

Effect of Intraperitoneal Injections of Histamine Acid Phosphate on Normal Rats.

Rat No.	Weight	Histamine acid phosphate per kg.	Results
	gm.	mg.	
94	165	1000	Recovered after few hrs. in good condition.
95	232	1200	" " " " " "
96	150	1300	" " " " " "
97	195	1400	" " 10 " " " "
98	185	1500	" " 10 " " " "
99	155	1600	Dead after 18 hrs.
100	170	1700	" " 11 "
101	188	1800	" " 4 "

5.5 mg. per kilo. Three rats injected at 30 days with 4.5 mg. per kilo were not killed. It appears therefore that suprarenalectomized rats at the height of susceptibility are about twice as susceptible

TABLE VII.

*Effect of Intravenous Injections of Histamine Acid Phosphate on Suprarenalec-
tomized Rats.*

No. of rats	Sex	Weight	Interval between operation and injection	Histamine acid phosphate per kg.	Histamine acid phosphate dose; amount injected	Duration of life after injection	No. of rats killed
		<i>gm.</i>	<i>days</i>	<i>mg.</i>	<i>mg.</i>	<i>hrs.</i>	
5	F.	165	20	298.1	49.2	10	5
	M.	200	20	406.0	81.2	$\frac{1}{2}$	
	"	220	20	491.7	108.2	$\frac{3}{4}$	
	"	220	20	245.9	54.1	$9\frac{1}{2}$	
	"	210	20	351.4	73.8	8	
5	"	220	7	346.3	76.2	5	5
	F.	170	7	520.6	88.5	$2\frac{1}{4}$	
	M.	220	7	171.2	37.9	Few min.	
	"	175	7	140.6	24.6	$6\frac{1}{2}$	
	"	225	7	141.7	31.9	3	
7	F.	165	6	123.0	20.3	6	7
	"	165	6	123.0	20.3	$4\frac{1}{2}$	
	M.	200	6	246.0	49.2	$2\frac{1}{2}$	
	"	200	6	246.0	49.2	6	
	"	210	6	199.0	41.8	18	
	"	240	6	205.0	49.2	3	
	"	230	6	164.7	37.9	$5\frac{1}{4}$	
3	F.	160	21	187.5	30.0	3	3
	"	165	21	181.8	30.0	12	
	"	175	21	171.4	30.0	18	
4	M.	225	6	80.0	18.0	3	3
	"	243	6	80.0	19.4	3	
	F.	180	6	80.0	14.4	$1\frac{1}{2}$	
	"	160	6	80.0	12.8	Survived.	
2	M.	250	6	60.0	15.0	"	0
	"	200	6	60.0	12.0	"	
1	"	240	6	40.0	9.6	"	0
4	"	285	6	20.0	5.7	"	0
	"	185	6	20.0	3.7	"	
	"	230	5	20.0	4.6	"	
	F.	175	5	20.0	3.5	"	
2*	M.	220	7	172.2	37.9	"	0
	F.	195	21	153.8	30.0	"	

* Controls—one adrenal intact.

TABLE VIII.

Influence of the Time Interval after Suprarenalectomy on the Effect of Intraperitoneal Injections of Histamine Acid Phosphate.

No. of rats	Sex	Weight	Interval between operation and injection	Histamine acid phosphate; amount injected*	Duration of life after injection	No. of rats killed
		gm.	days	mg.	hrs.	
6	M.	200	2	20.0	Survived.	1
	F.	165	2	16.5	2	
	"	200	2	20.0	Survived.	
	"	190	2	19.0	"	
	"	195	2	19.5	"	
	"	170	2	17.0	"	
5	"	220	3	22.0	"	2
	"	220	3	22.0	12	
	M.	250	3	25.0	Survived.	
	"	240	3	24.0	48	
	"	260	3	26.0	Survived.	
7	"	230	4	23.0	"	2
	F.	190	4	19.0	"	
	"	180	4	18.0	13	
	"	200	4	20.0	Survived.	
	"	165	4	16.5	"	
	"	190	4	19.0	"	
	M.	220	4	22.0	2	
12	"	190	5	19.0	2	12
	"	220	5	22.0	3	
	"	210	5	21.0	18	
	"	179	5	17.9	1	
	"	194	5	19.4	1	
	"	181	5	18.1	1	
	"	181	5	18.1	2½	
	"	201	5	20.1	½	
	"	193	5	19.3	3	
	F.	168	5	16.8	3	
	"	132	5	13.2	4	
	"	162	5	16.2	9	

* 100 mg. per kilo of body weight.

TABLE VIII—*Concluded.*

No. of rats	Sex	Weight	Interval between operation and injection	Histamine acid phosphate; amount injected*	Duration of life after injection	No. of rats killed
		<i>gm.</i>	<i>days</i>	<i>mg.</i>	<i>hrs.</i>	
10	F.	180	6	18.0	2	8
	"	200	6	20.0	18	
	M.	240	6	24.0	4	
	"	218	6	21.8	Survived.	
	"	240	6	24.0	1	
	F.	185	6	18.5	2	
	M.	210	6	21.0	Survived.	
	F.	159	6	15.9	1	
	"	150	6	15.0	2	
	M.	216	6	21.6	1½	
6	F.	200	7	20.0	Survived.	3
	"	210	7	21.0	"	
	"	160	7	16.0	2	
	"	225	7	22.5	1	
	"	190	7	19.0	Few min.	
	"	190	7	19.0	Survived.	
3	"	250	8	25.0	2	1
	"	180	8	18.0	Survived.	
	"	240	8	24.0	"	

to NaCN as are normal rats. This difference in the lethal dose of NaCN for suprarenalectomized and normal rats is, however, so small that this drug cannot be advantageously used as a test for decreased resistance. See Tables IV and V.

Histamine.—the increased susceptibility of suprarenalectomized animals to histamine has been shown by numerous investigators (8-12).

The intravenous M.L.D. of histamine acid phosphate for normal rats has been found to be approximately 900 mg. per kilo by Voegtlin (13) and 500-700 mg. per kilo by Crivellari (11).

In our experience (Tables VI,a and VI,b) the M.L.D. for normal rats is approximately 1600 mg. per kilo. As the preparation of histamine used in our experiments was the same as that employed in

the experiments of Voegtlin (13) and Crivellari (11) it is possible that the difference in the lethal dose is due to a difference in the age or in the susceptibility of the albino rats that we and they have used. Our rats were approximately 5 months of age and were reared in the laboratory from Wistar stock.

The increased susceptibility of suprarenalectomized rats to the intravenous injection of histamine is shown in Table VII. As the intravenous M.L.D. for these animals is approximately 80-100 mg. of histamine acid phosphate per kilo the suprarenalectomized rats are about 20 times more susceptible to histamine than the normal rats of the same age and strain. Banting and Gairns (10) found that suprarenalectomized dogs are about thirty times more susceptible to histamine than are normal dogs.

As the intravenous injection of histamine acid phosphate in doses as high as 2000 mg. per kilo involves the injection of comparatively large amounts of phosphoric acid, it seemed advisable to exclude by control experiments a possible effect of phosphoric acid. We therefore injected two normal rats with the amount of phosphoric acid per kilo contained in 2000 mg. of histamine acid phosphate per kilo. Instead of the phosphoric acid solution, an equivalent amount of $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ was used. The rats were sick for about 3 hours but recovered completely. Smaller amounts of $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ (1/10th of the dose used for the normal rats) had no effect on two suprarenalectomized rats.

The results of intraperitoneal injections of histamine acid phosphate in 49 suprarenalectomized rats are given in Table VIII. Of 18 suprarenalectomized rats injected with 100 mg. of histamine acid phosphate per kilo between the 1st and 4th days after the removal of both glands, 5 were killed, whereas 24 out of 31 suprarenalectomized rats injected at 5, 6, 7 and 8 days after the operation were killed.

A comparison of Tables VII and VIII shows that the difference in the M.L.D. of histamine acid phosphate for suprarenalectomized rats when given intravenously and intraperitoneally is negligible. Since the technique of intravenous injection in the rat is quite difficult, these results show that the intravenous method may be abandoned for this purpose.

We should like to call attention again to the latent period after

suprarenalectomy. As pointed out previously (6), the great drop in resistance does not occur until about the 5th day after the operation. This is illustrated in Table VIII where of 18 rats injected between the 2nd and 4th days, only 5 were killed, whereas of 31 rats injected with the same amounts between the 5th and 8th days, 24 were killed.

SUMMARY.

Of all the drugs thus far used in testing the resistance of suprarenalectomized rats, histamine has been found to be the most satisfactory. It is a readily obtainable and comparatively stable drug, producing characteristic symptoms. Its M.L.D. for a given strain of rats can be established within narrow limits, and the difference between the M.L.D. for normal and suprarenalectomized rats is comparatively great (1:20).

We wish to thank Dr. David Marine for his helpful advice and criticisms throughout the course of this work.

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LOCAL SPECIFIC THERAPY OF EXPERIMENTAL PNEUMOCOCCAL MENINGITIS.

IV. TYPE II PNEUMOCOCCAL MENINGITIS IN DOGS.

By FRED W. STEWART, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

PLATES 24 TO 28.

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This paper is the fourth of a series of studies on experimental pneumococcal meningitis. The three preceding (1-3) are concerned with various aspects, both pathologic and therapeutic, of experimental Type I pneumococcal meningitis in rabbits and dogs, together with a discussion of certain complicating factors in the disease. The pathologic studies are too extensive to summarize at this instance. From the point of view of therapy, however, it may be said that in one of these papers (2) a method was described whereby in suitable, uncomplicated cases, a number of permanent cures resulted. This method consisted in a saline lavage followed by a generalized flooding of the meninges with mixtures of immune serum and ethylhydrocupreine hydrochloride. The most important factor in obtaining successful recovery from the disease was our ability to bring repeatedly all infected regions of brain and cord into contact with the serum-drug mixture. This possibility, in turn, was favored by the nature of the early pathologic reaction on the part of the animal. With the pneumococcus strains employed, the animals reacted with only a moderate early leucocytosis; the fibrin was *relatively* scanty and there was a strong tendency of the spinal fluids toward the assumption of the picture which the French describe as "dissociation cyto bactériologique du liquide céphalo rachidien," *i.e.*, a fluid with many organisms yet a relatively mild fibrinocellular reaction. In other words, therapeutic possibilities were excellent. The exudate was not so fibrinous as to preclude extensive lavage and good serum-drug contact with the

pneumococcus. Although the organisms at first were present in very large numbers, after the lavage they were greatly diminished and following serum injection the degree of phagocytosis was very marked. As the disease progressed, *with or without treatment*, in certain of the animals a degree of partial immunity was apparently established and its expression was evidenced by a massive leucocytosis; this leucocytosis, however, was not very effective, since no matter how many leucocytes were present in the spinal fluids, the phagocytosis, in the absence of immune serum, was slight. This very leucocytosis, instead of favoring recovery under treatment, actually prevented effective treatment, since under these conditions lavage became impossible and injected serum failed to distribute itself properly. A successful treatment was one in which, by lavage, very early reduction in the number of organisms occurred, in which serum-optochin injections brought about active phagocytosis, and which, by repetition, prevented subsequent development of organisms to an extent sufficient to excite these massive purulent reactions.

It is a well established fact that the potency of most Type II anti-pneumococcus serum is low. Nevertheless, since in meningeal disease the therapeutic agent is introduced intrathecally into direct contact with the infecting agent, it was deemed reasonable to attempt the therapy of Type II meningitis in a manner similar to that employed in the treatment of Type I. It may be stated at the outset that we were unable to cure any of the animals. The reason for this failure will be readily apparent from the following description and discussion of the work.

Course and Pathology of Disease.

Four strains of Type II pneumococcus were used in the production of the disease. In all instances a Petroff-Hausser counting chamber served to determine dosage. As a rule 6 hour dextrose broth cultures were employed and the amount injected was usually from 0.5 to 1.0 cc. of a 1:100 culture dilution. No attempts were made to alter artificially by serial animal passages the "virulence" of the strains; every strain was transferred on receipt to blood broth and kept as stock, transplants being made to dextrose broth and incubated about 6 hours before use. Growth was always very heavy. One of the strains (II, 62) was of low activity in the dog, as appears from the following protocols.

Dog 1.—Female hound; weight 4 kilos. October 13, 1927, 4.35 p.m., cistern

puncture under ether; clear fluid. Injected 1.7 million Type II pneumococci, Strain II, 62. Normal ether recovery.

18 hours: Apparently well.

23 hours: Ether; cistern puncture; slightly cloudy fluid; rare cells and no cocci in smears. Culture negative. Subsequent uneventful recovery.

Dog 2.—Hound; weight 5 kilos. October 17, 1927, 4.00 p.m., cistern puncture under ether; clear fluid. Injected 10 million Type II pneumococci, Strain II, 62.

19 hours: Slightly sluggish; slight ataxia. Ether, lumbar and cistern taps; drained 3 cc. very cloudy fluid under tension from cistern; smear showed numerous polymorphonuclears per oil immersion field, but no cocci were seen. Upon planting a large amount of the fluid on a blood agar plate only fifteen colonies resulted. A spasmodic muscle contraction resulted in cisternal hemorrhage with death. Autopsy showed, aside from the area of hemorrhage, a grossly normal brain; no cocci were seen in any of the smears. Cultures from all regions were negative, except that a plate planted with the entire content of one lateral ventricle grew eight colonies.

Other animals infected with this strain made it appear that the strain activity was not such as to render its use desirable in our work. Some strains of Type II are apparently of even much lower activity, as demonstrated by the work of Idzumi (4). Idzumi infected his dogs subthecally with very large amounts of a Type II pneumococcus (8 cc. of a 24 hour broth culture lethal for mice in 50 to 60 hours in a 10^{-5} dilution). This dosage killed the dog in 20 hours. Only one experiment is reported and the pathological findings after this very large dose do not especially differ from our own, when using much smaller infecting quantities.

Two of our strains designated as D 39 and II 740 produced pictures identical in most respects. If anything, II 740 was somewhat more active. Bacteremia was rarely a problem; a few colonies from the postmortem heart's blood were a frequent finding. When significant bacteremia did occur, it was apparently the result of some individual peculiarity; it was—of course within reasonable limits—independent of weight of animal and infecting dosage, as the following two protocols demonstrate.

Dog 3.—Hound; weight 3.5 kilos. May 19, 1927, 10.45 a.m., cistern puncture under ether. Injected 18.0 million Type II pneumococci, Strain D 39. Anesthesia continued with intravenous sodium diethylbarbiturate. Samples of

blood were withdrawn at intervals from the leg vein and planted in 1 cc. quantities on blood agar plates with the following results:

1 hour.....	no growth.
3½ hours.....	no growth.
5½ hours.....	5 colonies per cc.

22 hours after infection the dog was found dead. Autopsy revealed generalized purulent meningitis; the entire brain was covered with yellowish, adherent, exudate. Abundant cloudy fluid in the cord meninges; cortex and cord showed petechial hemorrhages. Organisms were present everywhere, but never as numerous as one finds in the animal dying of Type I infection; especially in the cisternal pus there was very marked phagocytosis and one could observe many foci of pseudoagglutination. (We have coined the term "pseudoagglutination" to describe the appearance of exudates containing clusters of pneumococci formed by growth of groups of cocci recently liberated by the degeneration of leucocytes which had previously phagocyted them.) The heart's blood gave slight growth. The lungs showed congestion and edema.

Compare this with the following protocol of an experiment performed with the same culture, the same day, on a dog of the same weight and litter. The infecting dose was only half that used in the preceding case, yet there was early generalization of the disease.

Dog 4.—Hound; weight 3.5 kilos. May 19, 1927. 11.00 a.m., cisternal injection under ether of 9.0 million Type II pneumococci, Strain D 39. Anesthesia was continued with intravenous sodium diethylbarbiturate. Interval bleedings gave the following results:

1 hour.....	no growth.
3½ hours.....	no growth.
5½ hours.....	1050 colonies per cc.

At 22 hours the animal was found dead. Autopsy showed a dry, hyperemic brain; the exudate was scanty; cells and cocci were rare. In addition there were acute seropurulent pleuritis and peritonitis; pneumococci were demonstrated in the fluids both by direct examinations and by cultures. The heart's blood culture gave massive, confluent growth.

Only twice during the study of Type II meningitis have we felt that there was sufficient generalization of infection to modify the prognosis of the disease. These infecting doses given in the two preceding experiments were somewhat larger than those subsequently employed. In brief, infecting amounts of 18, 15, and 12 million organisms of the D 39 strain given intracisternally killed in less than 1

day. Smaller numbers have given more inconstant results. Doses of 7 million organisms in three experiments permitted survival up to 40, 42, and 66 hours respectively; however, all of these survivals received one or more treatments which may possibly have delayed death. Intracisternal doses of 3 to $3\frac{1}{2}$ million pneumococci usually allowed survival beyond the 1st day of disease, but even with this low dosage some animals have succumbed within 18 hours. The lower the infecting dose, the more apparent are the individual differences as regards the period of survival. Obviously a dosage which is quite apt to kill in less than 1 day is not a suitable one for the production of a type of disease which may be studied from the point of view of therapy.

Of course we can offer no explanation for individual differences in the survival period of the animals. There are, however, certain facts which correlate these differences with the pathological findings in the different animals. When the same amounts of the same culture are used for infection, it is impossible to see how the pathology can be explained by any difference in the infecting organism. The only variant is the response of the animal and the varying intensity of this reactional process is very important; this is naturally true in any infectious disease, but perhaps it is most apparent in central nervous system infections of the variety under study, because the nature and relations of the brain and cord make them unsuitable to withstand any severe, acute, reactional pathology.

If one infects a dog intracisternally with Type I pneumococcus and subsequently examines the spinal fluid, one notes, after a brief period during which time the organisms diminish somewhat in numbers, due in part to a slight initial phagocytosis, a progressive increase in organisms. Phagocytosis becomes negligible and the picture is one of cytobacteriologic dissociation. In Type II infections, from the start there is rather marked phagocytosis of cocci, even approaching in degree that seen after serum therapy of Type I. Coupled with this primary resistance to Type II there is a very heavy exudation of fibrin and polymorphonuclear leucocytes, and a tendency toward localization of the disease; this attempt at localization is never, in our experience, completely successful, but its effect may be disastrous to the animal, as the following protocol demonstrates.

Dog 5.—Male collie pup; weight 4½ kilos. September 27, 1927, 4.25 p.m., morphine; ether; cistern puncture; clear fluid. Injected 3.5 million Type II, Strain D 39. Normal ether recovery.

18 hours: Was just expiring when cage was visited. Autopsy: cord meninges edematous, injected, hemorrhagic, filled with fibrinopurulent exudate. Smears show numerous pus cells and a moderate number of diplococci, with evidence of considerable phagocytosis, coccal disintegration, with some small, atypical, and Gram-negative forms. Grossly about the medulla, cistern, and pontile base, the exudate is very thick and so fibrinous that it must be stripped off with forceps. The medullary smear resembles that from the cord. Convexity and base show much hemorrhagic injection (Fig. 1). Along the veins of the convexity is a moderate amount of cloudy fluid, containing fairly numerous cocci. The lateral ventricles contain abundant opalescent fluid, with rare cells but numerous cocci. There is slight bacteremia.

Fig. 2 gives some indication of the fibrinous character of the exudate at the site of injection. The fibrin distribution is that characteristic of those cases which die early after cistern infection; there is a very heavy layer closely surrounding the cord or medulla, and this layer continues out in similar fashion about the nearby vessels; this dense fibrin is comparatively devoid of cells, but further out, where the fibrin meshes are less dense, polymorphonuclear leucocytes are abundant. Curiously enough, by Gram-Weigert staining few pneumococci are found in the dense fibrin layers, but in the looser, fibrinocellular portion they are abundant. The vessels may show considerable damage and frequently, from serum exudation into their walls, they stain in a hyaline manner; the vessel walls are extensively infiltrated by polymorphonuclear leucocytes. Occasionally with these marked reactions it is possible to detect deep changes in the nerve cells of the cord. These changes consist in necrosis with polymorphonuclear reaction and invasion of the affected nerve cells (Fig. 11). The smaller vessels of the gray matter show some polymorphonuclear perivascular reaction, and there is slight generalized, or patchy infiltration (Fig. 12) of the interstitial nerve tissue either in the cornua or fasciculi. This is not the same phenomenon which one sees in a spreading myelitis of central canal origin, for in the former it has not been possible to demonstrate organisms in the region or in the finer vessel sheaths, whereas in the latter—the central myelitis—organisms are very abundant and the pathological picture is quite different. The pathology suggests the action of a diffusible toxic substance; were it not for the marked

meningeal reaction, one might easily confuse the picture with poliomyelitis. In the protocol of Dog 5 it is seen that the organisms were rather numerous; the infection at death was severe; changes in deep nerve cells may have hastened death, but in other animals death has occurred without a marked proliferation of organisms, without massive ventricular growth, without detected deep nerve cell pathology, and in the absence of significant bacteremia. Nothing, therefore, is left to account for death, except the presence of the massive exudate in the cisternal region adjacent to vital centers, and experience forces us to attribute to this reaction very unfavorable significance. We noted in Type I pneumococcal meningitis the severity of symptoms for a few hours after extensive serum treatment and were inclined to attribute it to the marked increase in leucocytosis—a transient phenomenon. Had a general anesthetic not been necessary, we would have drained our animals several times at the height of the leucocytosis to relieve the intensity of the reaction and it is suggested that in the treatment of human disease this procedure would be desirable.

The effect of a massive cisternal exudate is apparent in another way. The space is small and a block is readily attained. As a rule, the lateral ventricles are infected early in the course of the disease. A primary cisternal injection in the amount used in our experiments does not pass into the lateral ventricles; as judged by dye injections in equivalent quantities, it distributes itself to the cervical cord, fourth ventricle and its meninges, and to the base and adjacent lower lateral portions of the cerebrum; the cerebellum may be stained, but the tentorium prevents forward spread to the convexity. Apparently infection follows the same course. A marked early, cisternal block may occur, with organisms present in the cisternal exudate but without cocci in the lateral ventricles at the time of death. In the absence of early block the ventricles may become infected very soon in the course of the disease, and in this case the pneumococci multiply to an extraordinary extent in the lateral ventricles behind the subsequently developing block, and fairly well established hydrocephalus may develop within 2 days after primary infection. An interventricular block may result in quite different pictures in the two lateral ventricles. The following two protocols show the probable effect of cisternal block on the distribution of the infecting agent.

Dog 6.—Hound; weight 8.4 kilos. October 24, 1927, 4.35 p.m., ether; cistern puncture; clear fluid. Injected 5 million Type II pneumococci, Strain D 39.

20 hours: Dead. Autopsy reveals the usual fibrinohemorrhagic exudate blocking the fourth ventricle and extending over medulla and cervical cord; small amount of purulent exudate at the base; lower cord and convexity practically clear in gross; the lateral ventricles contain abundant opalescent fluid with pus cells but no cocci; cervical cord and cisternal smears show abundant pus cells and a few free and phagocytosed cocci. A "reactional" death.

Dog 7.—Fox terrier; weight 5 kilos. October 19, 1927, 4.15 p.m., ether; cistern puncture; clear fluid. Injected 2½ million D 39 Type II pneumococci.

18 hours: Dead. Autopsy reveals much fibrin and some clot in fourth ventricle. Cord and base show moderate purulent exudate. Softening in posterior vermis. Cortex dry and injected. Abundant cloudy fluid in lateral ventricles. All smears show numerous pus cells, but the cocci are rare and nearly all phagocytosed, except in the lateral ventricles, where they occur free in enormous numbers (Fig. 5). Cultures from lateral ventricles yield confluent growth; from other regions a few colonies only. Slight bacteremia.

Block is of importance in influencing the distribution of organisms, especially when it occurs in the cisternal region; its influence in other situations has not been determined, but is probably less. Its significance at the cisterna is most apparent when infection passes the early block and invades the lateral ventricles, for it has been abundantly demonstrated that in the ventricles with a favorable fluid medium the organisms grow to an enormous extent, whereas in less favorable regions, base, cord, and convexity, there is some tendency to spontaneous disappearance partially, at least, through phagocytosis. In the lateral ventricles the rapid growth of pneumococci seems to repel phagocytosis and it is not seen save to a minimal degree in the absence of immune serum. Figs. 6 and 7 are from the autopsy of a dog infected in the lateral ventricle. The animal died in 18 hours; there was no blocking exudate in the cistern; the foramina of Munro may have been occluded and this is probable since the picture from the two lateral ventricles was not the same. Some organisms, however, have passed through into the cistern and meninges, and the smears, as is seen, are quite different; in one there is abundant growth and little or no phagocytosis, whereas in the other—from the cistern—phagocytosis is marked and cocci are few. Therefore, it is apparent that even without block the rapid proliferation of organisms in the lateral ventricles fades out as the cisternal and cord regions are reached. What block

does is to delay lateral ventricle infection, if the block occurs early, and to place a barrier between a site of prolific growth and a region of much less active multiplication, if it occurs only after the ventricle is infected. Some of these cells in the cistern smear (Fig. 6) are endothelial leucocytes. It is easily shown that the endothelial leucocyte readily phagocytes Type II pneumococci in the dog. This was particularly apparent in the case of a dog infected in the basal region; the animal developed a pneumococcus brain abscess along the needle track (Fig. 9). Many endothelial leucocytes were attracted to the softening areas at the periphery of the abscess, and all of these are loaded with pneumococci, many of which are fragmented. Toward the center of the abscess, where there are only polymorphonuclear leucocytes, pneumococci are abundant and there is little or no phagocytosis. When softening develops, as it may in foci in a meningeal exudate, endothelial leucocytes may appear at the periphery of softened areas and actively phagocyte pneumococci; in these rare foci of softening in the meningeal exudate, however, the pneumococci tend to disappear; they are probably injured by the mass liberation of ferments from the degenerated leucocytes.

We have discussed the influence of the massive fibrinopurulent exudate in the cisterna on life and on the distribution of infection. It is interesting to note the effect of this type of reaction in other regions. The very large fibrin content of the exudate tends to be maintained everywhere, except in the lateral ventricles, where either the process results in a purulent reaction, or a type resembling the "dissociation cyto-bactériologique" common in Type I. Next to the cistern the penetrating vessels suffer most markedly from the character of the exudate. This is apparent, though by no means uniform, in several regions,—cord, convexity, and thalamus. We have followed the process by ordinary methods and by Gram-Weigert staining, and are inclined from our observations to interpret it as follows: Organisms invade the penetrating vessels and their perivascular sheaths from the meninges. The reaction consists in marked polymorphonuclear leucocytic invasion of the vessel walls and an exudation of serum, fibrin, and leucocytes into the sheaths and surrounding parenchymatous tissue. The reaction takes place in the vessel walls and perivascular sheaths in advance of detectable invasion by the pneumococci, and it

is possible to observe, by Gram-Weigert staining, pneumococci to a certain level, marked reaction somewhat deeper still, and not infrequently thrombosis of the smaller branches. Fig. 13 illustrates the process in the deeper cord vessels; Figs. 14 and 15 in the thalamus; in the latter a smaller branch is thrombosed and resultant changes in the surrounding parenchyma have attracted leucocytes into the brain tissue. This region as yet is apparently uninfected, but when organisms finally do penetrate into the focus, they proliferate rapidly in the injured brain tissue and readily convert an area damaged by thrombosis into a pneumococcus abscess. In the cord region central myelitis is common. Vessel sheaths are early involved by the infectious process originating from the central canal (3) and central softening occurs; a similar type of lesion spreads in along vessels, penetrating from the meninges, and the combination destroys large portions of spinal cord. In the central myelitis the first organisms reach the cord tissue through the ependyma (Fig. 16). Once in the parenchyma they invade extensively between the swollen, damaged, myelinated fibers (Fig. 17).

Often considerable hemorrhage is present. When this occurs, it is usually in the cisternal region and for a while, although our initial cisternal taps at the time of infection gave clear fluid, we were inclined to attribute it to trauma. Later, however, it was noted in other regions, notably choroid plexus and convexity. Fig. 10 gives some notion of the extreme vasodilatation one may encounter in the choroid plexus of the lateral ventricles.

All of the preceding description refers to the sequences of primary cisternal infection. We were forced to abandon this method of inoculation because of the severity of the local reactions. Other methods of infection were next employed. One animal was infected in the lateral ventricle in the hopes that the infection would be distributed more gradually *via* the usual cerebrospinal fluid pathways, thus avoiding early severe, local reaction in the cisternal region; the method was worse than useless, for not only did it fail to prevent rapid spread to the cistern, but in addition it placed the pneumococci immediately in that region in which we have determined their growth to be most rapid and luxuriant. Fig. 7 illustrates the condition 18 hours later in the lateral ventricle. Infection directly through the brain to the

base was attempted in several instances. It was a first impression that this produced a more favorable type of disease, but in reviewing the records this opinion scarcely seems justified. Organisms introduced into the basal meninges do not tend to proliferate markedly at that site, or to spread in large numbers to the convexity. Instead they penetrate to the cisterna and thence to lateral ventricle and cord. In making this statement, we cannot be certain that initial lateral ventricle infection has not occurred along the needle track. Our assertion is merely based on numbers of organisms in different regions. In basal infection brain abscess may occur along the needle track. Primary infection of the convexity produced a severe clinical picture with marked depression, opisthotonos, spasticity of all four limbs, vomiting, and respiratory difficulty within 20 hours after infection. Death occurred during the 2nd night; the meningitis was already generalized, fibrinopurulent in character, but, although death occurred early, organisms were exceedingly rare and largely phagocytized. Death, we believe, was "reactional." Attempts at infecting the animals in the lumbar meninges are best taken up with our discussion of therapy. Infection by the lumbar route spreads more slowly than it does by the other routes employed.

Therapeutic Experiments.

The method employed in treating the animals which survived long enough to make a therapeutic effort feasible was the same as that already described for the treatment of Type I meningitis (2). Studies of the distribution of the process made us, however, abandon direct serum-drug treatment of the convexity since that region, due to localizing efforts on the part of the animal, was only rarely involved. As we have stated, the lateral ventricles early become the chief reservoir of pneumococci; lateral ventricle punctures were therefore used rather extensively both for drainage and serum-optochin injections. The serum employed was kindly furnished by Dr. Wadsworth, of the New York State Department of Health. The following series of protocols are offered as examples of therapeutic experiments. It will be readily noted that the possible degree of success apparent in some instances is negated by the complete failure in others. Indeed, it may be stated

that treatment appeared without value, even when it was carried out so early in the course of the disease that diagnostic punctures at the time the treatment was initiated not only failed to reveal pneumococci in the fluid smears, but likewise in cultures. If success had been attained in such instances, it would be of little significance, since no human case could possibly be diagnosed and typed at so early a period and the disease is one in which a few hours of progress make a profound difference in the picture.

Dog 8.—Hound; weight 4½ kilos. November 2, 1927, 4.30 p.m., morphine; ether anesthesia; skull trephine; puncture through to left base; slight hemorrhage. Injected 3½ million, Type II, D 39 strain. Normal recovery.

18 hours: Sluggish; chills; no meningeal signs.

24 hours: Still stuporous from morphine. Has slept all day. On disturbing, stands up, walks, and wags tail.

42 hours: Apparently well. No treatment.

66 hours: Very ill; irritable; lies prostrate on side; all extremities spastic with exaggerated reflexes. Lumbar and cistern puncture under ether. No fluid obtained *via* lumbar needle, but withdrew 1.5 cc. very thick purulent exudate of gelatinous consistency from cisterna. A smear showed large numbers of polymorphonuclear leucocytes and pneumococci (Fig. 4). On account of the thickness of the exudate, no saline lavage was possible. The usual serum-optochin mixture was prepared (10 cc. serum plus 0.5 cc. 1 per cent ethylhydrocupreine hydrochloride) and 4 cc. were injected, half in the cisterna and half in the lumbar region. After a few minutes rest both lateral ventricles were entered through trephine openings and the contents aspirated. Apparently the immune serum injected into the cisternal region had already reached the lateral ventricles, since the abundant pneumococci were all agglutinated (Fig. 8); cells were very scanty; 0.6 cc. serum-optochin injected in each lateral ventricle and 0.6 cc. over each convexity. Recovery rate difficult to detect on account of initial prostrate condition. Placed in constant body temperature cage and removed to animal house after some hours.

90 hours: Dead. Autopsy shows heavy fibrinopurulent and hemorrhagic exudate over cord and about cistern; exudate scanty at base, where initial infection was introduced. Small amount of pus in lateral ventricles. Smears from all regions show very numerous pus cells; cocci are diminished in numbers (Fig. 3) and where numerous show agglutination. There is little evidence of stimulated phagocytosis. Small abscess of medullary floor (traumatic?). Beginning hydrocephalus.

This animal probably had his pneumococci reduced by the treatment, but it is difficult to ascertain truly if this was the case. Other experiments at least throw considerable doubt into the matter.

Dog 9.—Hound; weight 4½ kilos. September 29, 1927, 4.20 p.m., morphine; ether; cisternal tap; clear fluid. Injected 1½ million Type II D 39. Normal recovery.

18 hours: Temperature 102.7°. Stupid and refuses to stand up. Raises head and is irritable when touched.

21½ hours: Ether; cistern and lumbar punctures; fluids cloudy; contain 25–30 polymorphonuclears per oil immersion field; no cocci seen in direct smear. Cultures: growth but not confluent. Lavage of 5 cc. NaCl, lumbar to cistern, with good return. Distributed 11 cc. serum-optochin mixture as follows: 8 cc. cord to cistern, 1.5 cc. frontals *via* trephines. Satisfactory recovery.

44 hours: Temperature 102°. Has lost weight; condition otherwise unchanged. ether; lumbar and cistern punctures; fluid clearer; cells rare; many mononuclears; no cocci seen. Injected 5 cc. serum-optochin *via* cord and cistern. Cultures negative.

Subsequent uneventful recovery.

The above protocol shows the influence of treatment but only in a prophylactic way. At the time therapy began the case was not capable of being diagnosed. As we have intimated, even as a prophylactic, the serum may fail to do anything save to delay the inevitable, as the following example clearly demonstrates.

Dog 10.—Weight 5½ kilos. December 5, 1927, 4.30 p.m., ether; lumbar puncture; clear fluid; injected 2 million Type II pneumococci, Strain D 39. Good recovery.

19 hours: Weakness in lumbar region; slight tremors in hind limbs; otherwise normal.

23 hours: Unchanged. Ether; lumbar puncture; cloudy fluid; cells 15–20 per oil immersion field; no cocci seen. Cisternal fluid clearer; few cells and no cocci. *Neither fluid gave a positive culture.* Lavage with 10 cc. NaCl lumbar to cistern; return unsatisfactory, but no pressure symptoms. Injected as usual 10 cc. serum. (No drug was used, since without much exudate and with poor fluid return there is great danger.) Both lateral ventricles were entered and, after withdrawal of a practically negative fluid already mixed with serum, 1 cc. of additional serum was introduced. Lateral ventricle culture gave no growth.

42 hours: Unchanged. No treatment.

66 hours: No treatment. Appears well.

7th day: Has suddenly become very ill. Necessary to chloroform. Immediate autopsy. Massive, generalized meningitis; complete block at the cisterna; hemorrhagic flecks over convexity. Every smear shows very numerous pus cells. Cocci are very abundant in the lateral ventricles, numerous in the cisterna, few at the base, and very few over the convexity and in the cord meninges. The heart's blood yields rare colonies.

Summary: Cord infection; extensive early treatment when organisms were so few that they were not detected by culture, yet the fatal outcome of the disease was merely delayed.

The objection may be raised that treatments in cases such as that summarized above were not systematically continued. In the absence of organisms in the initial cultures and smears, it would have been quite illogical to treat. In the following protocol the effect of repeated treatments is shown.

Dog 11.—Hound; weight 6 kilos. November 21, 1927, ether; left frontal trephine; puncture through to base and injection of 2 million Type II pneumococci, Strain D 39. Normal recovery.

18 hours: Ataxic; irritable; able to stand. Morphine; ether; cistern and lumbar punctures; fair drainage; fluids opalescent, containing on smear about 25 polymorphonuclears per oil immersion field; cocci very rare. Injected 3 cc. serum-optochin cord, 2 cc. cistern, 0.6 cc. right lateral ventricle, 1 cc. left lateral ventricle, and 0.5 cc. subdurally over each convexity. Some shock but recovery in 45 minutes. Cultures positive.

42 hours: Clinically unchanged; possibly slightly improved. Cistern and lumbar punctures under ether. Fluids less cloudy; cells diminished but cocci slightly more numerous; they are both free and phagocytosed, and occur singly and in rare clusters of 5 or 6. Injected 4 cc. serum-optochin cord, 1 cc. cistern, and, after draining, 1 cc. in each lateral ventricle; smears from the ventricles show occasional free cocci. Cultures yield confluent growth.

66 hours: Unchanged except for loss of weight. Ether; cistern and lumbar punctures; fluid slightly clearer; cells diminished; no cocci seen. Lavage with 5 cc. serum-optochin lumbar to cistern; lumbar needle removed and 1 cc. additional injected into cistern. Left lateral ventricular puncture; fluid practically clear; smear shows rare cells but no cocci. Injected 0.5 cc. serum-optochin. Cultures give slight growth.

90 hours: Animal worse; more irritable. Ether; no cistern fluid obtained; no lumbar fluid. Nevertheless, 3 cc. of serum-optochin was injected. The left lateral ventricle was entered and 0.5 cc. of what appeared in gross as clear serum was withdrawn. 1 cc. serum-optochin injected. Smears of this apparent serum show essentially no cells but numerous clusters of agglutinated pneumococci. Culture yields a confluent growth.

114 hours: Prostrate; convulsive; very hypersensitive. Chloroformed. The usual autopsy findings. Massive fibrinopurulent exudate in and about cistern, at base, and in lateral ventricles. Cord and convexity are relatively clear. Smears show hundreds of leucocytes and pneumococci per oil immersion field. Little phagocytosis except in cord and cisternal regions. Hydrocephalus. Multiple brain abscesses along basal and lateral ventricular needle tracks.

In other words, systematic treatments extended over several days failed to cure a very slight initial infection. It undoubtedly did delay death.

SUMMARY AND CONCLUSIONS.

1. With properly selected strains of Type II pneumococci, fatal meningitis may be produced by intrathecal infection in dogs.
2. The pathology of the disease differs from that produced by Type I pneumococci, at least with the strains employed.
3. Type II meningitis is characterized by early, very marked reactions, a very heavy fibrin exudation, and a tendency toward the rapid establishment of blocks.
4. Extreme vasodilatation and hemorrhage are common.
5. The fibrinopurulent exudate tends to involve the walls and perivascular sheaths of deeply penetrating vessels, thereby causing thrombosis.
6. Thrombosis leads to foci of parenchymatous softening which, on subsequent infection, become brain abscesses.
7. The lateral ventricles are a "point of election" for the luxuriant growth of pneumococci. Conversely there is evidence that Type II organisms tend somewhat to disappear from other regions, owing either to spontaneous phagocytosis or to filtration into the blood stream. The lateral ventricles, however, continually renew the supply.
8. Hydrocephalus occurs early—sometimes during the 2nd day of the disease.
9. Central myelitis is common and appears early in the disease.
10. Deep nerve cell lesions with necrosis and neuronophagia are encountered. Their pathology suggests a toxic source.
11. In most cases treatment has been impossible owing to severe early reactions, and only as a prophylactic has optochin-serum therapy any real value. It delays death, but has in no instance cured established disease in the dog.
12. Systemic generalization of Type II meningeal infection in the dog occurs, but it is rarely of sufficient extent to modify prognosis.

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EXPLANATION OF PLATES

PLATE 24.

FIG. 1. Lower aspect of brain. Fibrinopurulent exudate beneath dura, extending over cervical cord and forward toward infundibulum.

FIG. 2. Dog 5. Typical massive, fibrinopurulent exudate. Weigert stain. $\times 100$.

PLATE 25.

FIG. 3. Dog 8. Autopsy smear from cisterna. Gram stain. $\times 1000$.

FIG. 4. Dog 8. Initial smear from cisterna. Gram stain. $\times 1000$.

FIG. 5. Dog 7. Autopsy smear from lateral ventricle. Gram stain. $\times 1000$.

FIG. 6. Dog 12. Autopsy smear from cisterna. Gram stain. $\times 1000$.

PLATE 26.

FIG. 7. Dog 12. Autopsy smear from lateral ventricle. Gram stain. $\times 1000$.

FIG. 8. Dog 8. Agglutination of pneumococci. Gram stain. Lateral ventricular tap. $\times 1000$.

FIG. 9. Needle track abscess. Dog 11.

FIG. 10. Prehemorrhagic dilatation of vessels of choroid plexus of lateral ventricle. Hematoxylin-eosin stain. $\times 40$.

PLATE 27.

FIG. 11. Dog 5. Spinal cord. Necrosis and early neuronophagia of motor nerve cells. Hematoxylin-eosin stain. $\times 100$.

FIG. 12. Dog 5. Spinal cord. Leucocytic infiltration of white matter in region of radix descendens V. Hematoxylin-eosin stain. $\times 100$.

FIG. 13. Dog 13. Spinal cord. Deep vascular lesions; polymorphonuclear invasion of vessel wall and serofibrinous exudation in and about vessel sheath. Hematoxylin-eosin stain. $\times 100$.

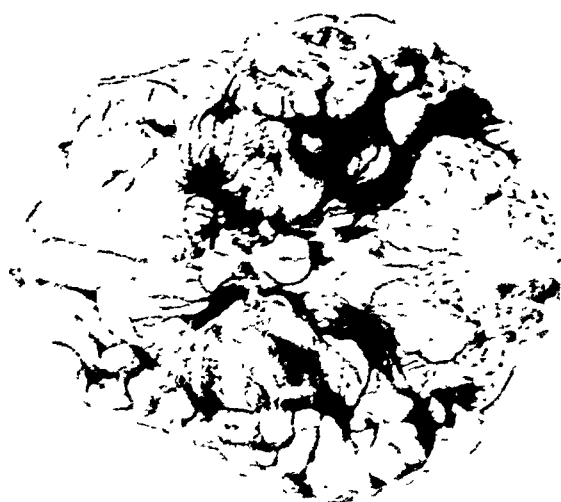
PLATE 28.

FIG. 14. Dog 14. Thalamus. Deep perivascular lesions. Hematoxylin-eosin stain. $\times 20$.

FIG. 15. Same. Perivascular invasion of white matter following thrombosis. Hematoxylin-eosin stain. $\times 300$.

FIG. 16. Dog 15. Early invasion of region of commissures by pneumococci. Gram stain. $\times 1000$. C = central canal of cord.

FIG. 17. Pneumococci infiltrating between myelinated nerve fibers just outside a region of purulent central myelitis. Gram stain. $\times 1000$.



1



(Stewart Intraocular Infection, IV.)

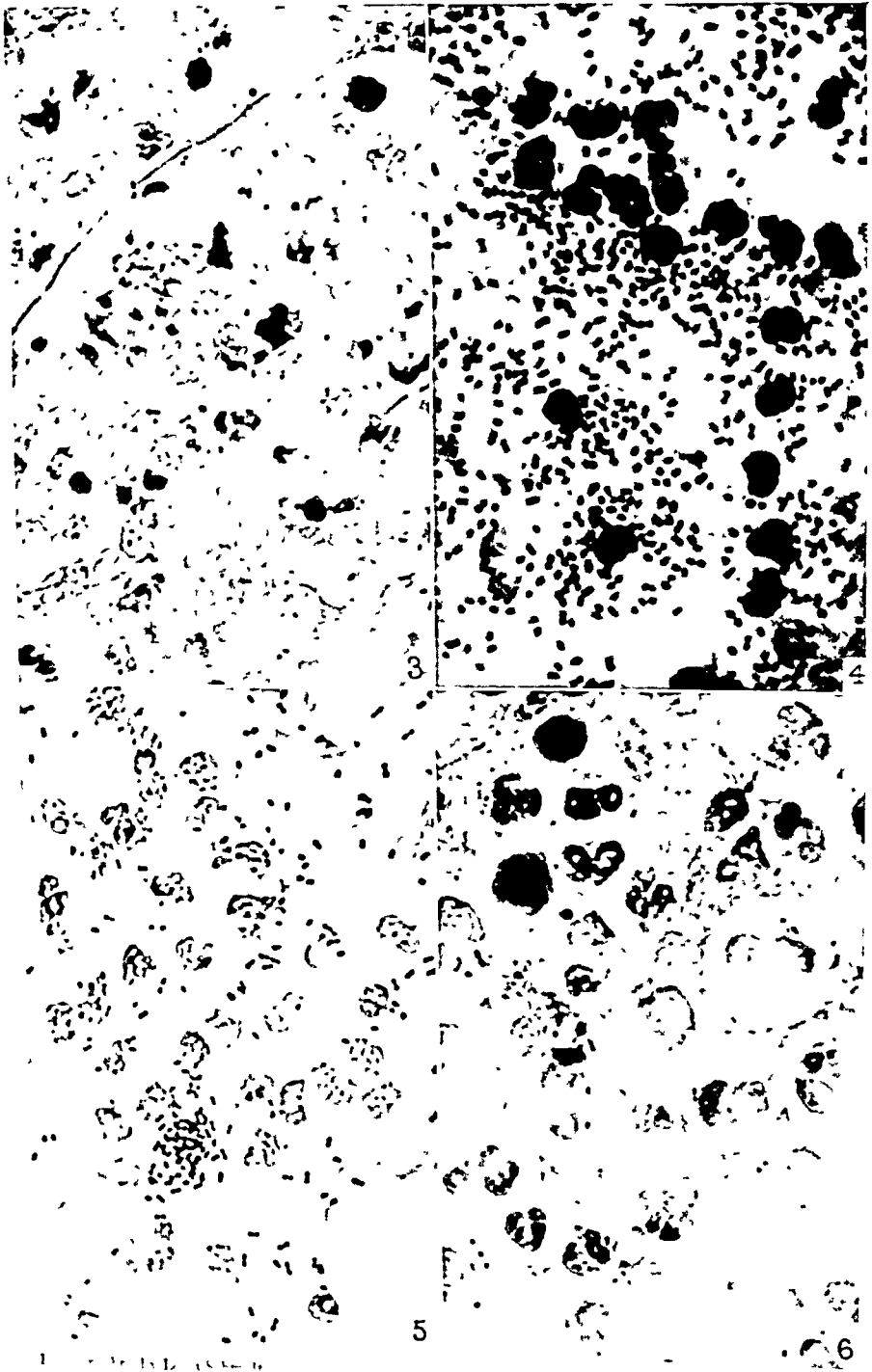
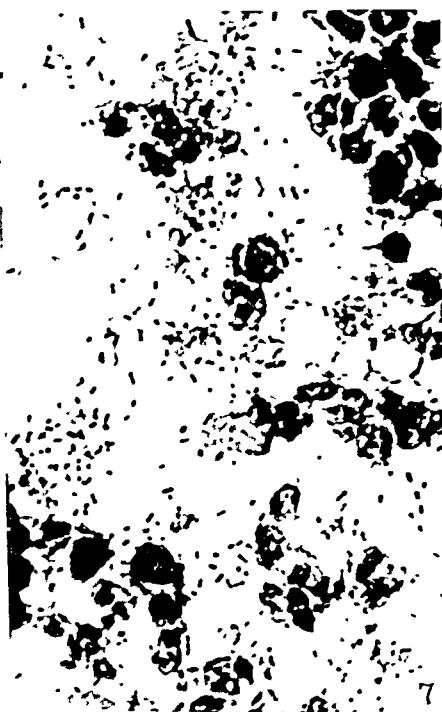


Fig. 1. (A) (B) (C) (D)

Stenohyal Pneumococcal meningitis (IV)



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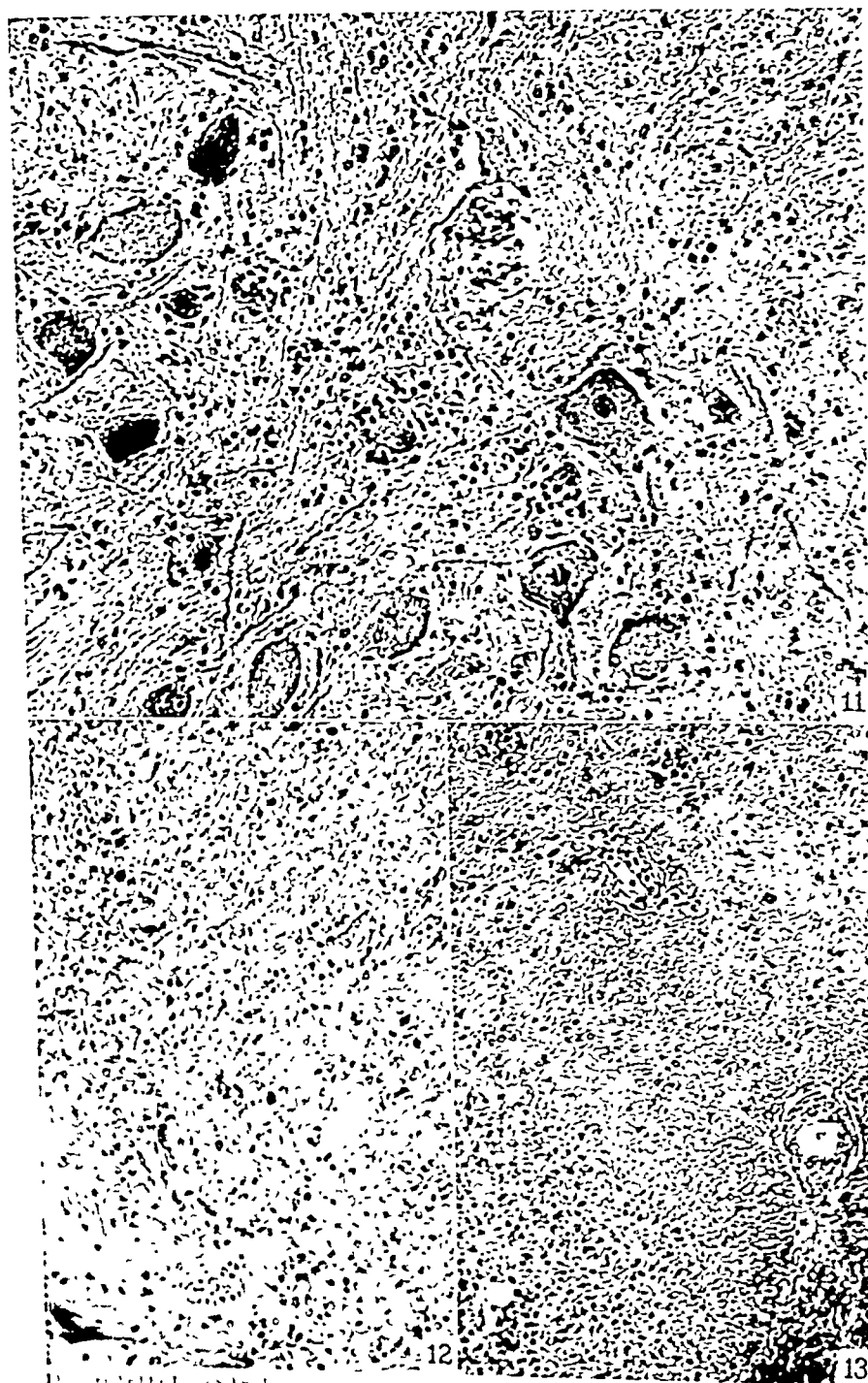
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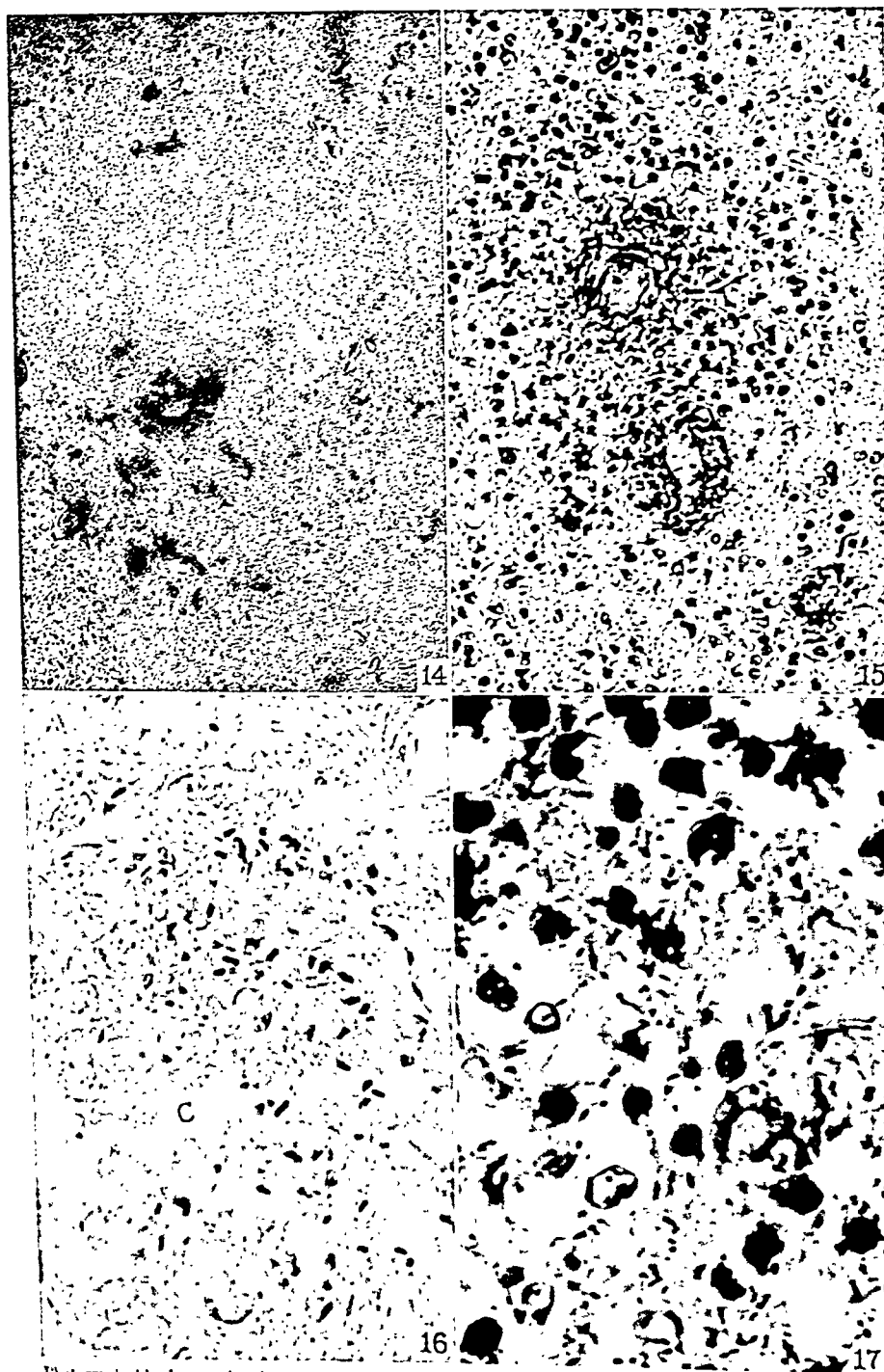


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Photographed by Louis Schwartz

(Stewart. Pneumococcal meningitis. IV.)

THE PRODUCTION OF SKIN NECROSIS BY CERTAIN AUTOLYSATES OF PNEUMOCOCCUS (TYPES I AND II).

By JULIA T. PARKER.

*(From the Department of Pathology of the College of Physicians and Surgeons,
Columbia University, New York.)*

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Many studies have been made on the various toxic substances obtained from pneumococci. Rosenow (1) and Cole (2) investigated the anaphylactic-like reactions produced in guinea pigs by the intravenous inoculation of autolysates of pneumococci and of the peritoneal exudates of animals dead of pneumococcus septicemia. The hemotoxin of the pneumococcus has been studied by Cole (3), Avery (4), and Neill (5), and the purpura-producing principle of the autolysates by Julianelle and Reimann (6). The effect caused by these various products of the pneumococcus when injected into the skin of man and animals has also been studied (7). No mention, however, has been made of the production of skin necrosis by sterile pneumococcus products, except by Zinsser (8), who was able to produce necrosis in the skin of large normal guinea pigs and in small guinea pigs which had been previously sensitized to the pneumococcus, by the intracutaneous inoculation of certain heat-stable aerobically produced autolysates of this organism.

When rabbits or guinea pigs are given virulent pneumococci (Type I) intradermally in doses so adjusted that the animals die in 24 to 48 hours, there usually develops at the site of inoculation an area of greenish necrosis several cm. in diameter. Except for its greenish color, the appearance of such a lesion is very similar to that produced by the intracutaneous inoculations of the staphylococcus exotoxin (10), and, therefore, it seemed possible that the pneumococcus lesion might be caused by the same kind of poison. The experiments to be described deal with some investigations made to determine the nature of this necrotizing agent.

EXPERIMENTAL.

The strain of *Pneumococcus* I used in this work was isolated in November, 1924, from the blood of a patient with lobar pneumonia at the Presbyterian Hospital. Since then it has been passed through rabbits or guinea pigs at least once in every 2 weeks and is now of such virulence that 1-10,000,000 cc. regularly kills rabbits. In mice the lethal dose is 1-1,000,000 cc. No higher dilutions were tried.

Numerous preliminary experiments were conducted to find out whether the necrotizing toxin could be obtained from filtrates of various aerobic and anaerobic broth cultures, exudates, and the expressed fluids of organs and tissues of rabbits and guinea pigs which had died of pneumococcus infection, but the results were uniformly negative.

We next began a study of the autolysates of pneumococci for the presence of this hypothetical necrotizing poison. In general, the method used was to take up the sedimented pneumococci from well centrifuged broth cultures in saline solution, pneumococcus broth, or other fluid, and to allow the suspensions to autolyze, with and without a vaseline seal, at room temperature for different periods of time. It was soon observed that a slight necrotizing action was sometimes obtained with the preparations sealed with vaseline, while the preparations kept without the vaseline seal never possessed this property. From this we inferred that the poison either was formed only in the absence of oxygen, or if formed, was destroyed by oxidation. Various methods of protecting preparations from oxidation were tried, with methylene blue as an indicator. It was found that the addition of sodium hydrosulfite (5), a reducing agent, interfered with the formation of the poison; and that the addition of other reducing agents, such as cysteine (9), live *B. coli*, or yeast, was of no apparent advantage in obtaining an active autolysate. Below I have described in some detail the method which is now being used for the preparation of this toxin.

Preparation of the Necrotizing Poison.

The pneumococci are grown on double strength veal infusion broth containing 4 per cent Witte peptone and 0.5 per cent salt. Flasks are filled nearly to the top with the medium which has been brought to a pH of 7.8. The medium is sterilized in the Arnold sterilizer by the intermittent method.

Each flask containing 200 cc. was inoculated with 1 large loopful of blood from the heart of a guinea pig or rabbit which had died of a pneumococcus infection. After 18 to 24 hours in the incubator, the broth cultures were chilled and then centrifuged at high speed. After centrifuging, they were chilled, the supernatant fluid poured off carefully, and the pneumococci taken up in a quantity of freshly boiled and chilled broth equal to that of the sediment with the small amount of supernatant fluid remaining. For pneumococcus sediment from 100 cc. of broth culture, there should be in all approximately 1.5 cc. of fluid. The pH of the pneumococcus suspensions is brought to 7 or 7.2 and cultures taken to make sure that only pneumococci are present in the preparation. The pneumococcus suspension is distributed into narrow test-tubes, which are then chilled for at least 30 minutes. After this, any bubbles present on the surface of the suspension are gotten rid of with a hot platinum loop and heavy vaseline seals added to all the tubes. The tubes are left at room temperature in the dark at 22-24°C. for 6 to 8 days and then placed in the ice box until used. Immediately before use, the autolysates are centrifuged, iced, the seals opened, and the clear supernatant fluid filtered through a well iced Berkefeld apparatus. This filtrate contains the necrotizing poison. It is necessary to keep the preparations chilled when they are exposed to the air, otherwise they become oxidized and the toxicity disappears.

Young guinea pigs weighing from 250 to 350 gm. were inoculated intradermally on the side with 0.1 cc. of sterile pneumococcus necrotizing poisons. The reaction to the intracutaneous inoculation in these animals is as follows: 10 to 30 minutes after the inoculation, a small purplish area, which gradually increases in size, appears at the site of inoculation. With strong poisons, this area covers a zone of 2 to 3 cm. in diameter within 2 to 4 hours. At this time the area usually appears purplish black, with an encircling border which is bright red in color and which appears to be hemorrhagic. 18 to 24 hours after the inoculation, the central purple zone has become definitely yellowish and necrotic. Several days later, this necrotic area has become a brown scab.

Properties of the Necrotizing Poison.

When filtration of an active autolysate is carried out rapidly through a new N or V Berkefeld filter, with every precaution taken to have the apparatus and autolysate well chilled, there is practically no loss of its activity. Such a filtrate will remain active for several weeks if it is sealed with vaseline immediately after filtration and preserved in the ice box. If opened, even when kept packed in ice, it quickly deteriorates.

The necrosis-producing principle of an autolysate is completely destroyed when heated under vaseline seal at 60°C. for 5 minutes. Under these conditions, with a pH of 6.8 to 7, there is usually a slight clouding of the filtrate.

Are the Pneumococcus Hemotoxin and Necrotizing Poisons Related?

There is no doubt that our necrotizing autolysates contain other pneumococcus products such as the poison which produced the anaphylactic-like symptoms, the hemotoxin, and the purpura-producing principle. Is, then, the necrotizing toxin identical with one of these previously recognized poisonous substances which has been demonstrated by other methods? Apparently, the only toxic product of the pneumococcus which need concern us is the hemotoxin which, as regards thermolability, sensitiveness to oxidation, and ability to be neutralized by immune serum prepared against it, appears to be very similar to the necrosis-producing poison. During the course of this work we have titrated many of our autolysates for the presence of the hemotoxin, and have found, as would be expected, that they always contained more or less of the hemolytic substance, but there was no parallelism between the amount of hemotoxin and of the necrotizing substances present in an autolysate. Moreover, the extracts produced by the freezing and thawing method of Cole and Avery, while rich in hemotoxin, contained little or no necrotic activity. Furthermore, we have been able to separate the hemotoxic and necrotizing substances by treating the autolysate with the red cells of rabbits or guinea pigs. By this procedure the hemotoxin is entirely removed by the red cells, while the necrotizing poison remains unaffected in the supernatant fluid. These last experiments seem to prove conclusively that the hemotoxin and necrotizing poison are different entities. An experiment showing the selective adsorption of the hemotoxin is described below.

This autolysate caused complete hemolysis of 2.5 cc. of a 1 per cent suspension of washed rabbit red cells in a dilution of 1-200 and 50 per cent hemolysis in a dilution of 1-1000. 0.4 cc. of washed rabbit red cells was thoroughly mixed with 2 cc. of chilled necrotizing filtrate. The mixture was allowed to stand for 4 minutes at 2°C. and then centrifuged at high speed for 5 minutes, care being taken to have ice water in the centrifuge cups. After centrifuging, the cold clear slightly green-

ish supernatant fluid was pipetted off and used along with the original filtrate in the tests for hemotoxin. Table I shows the results of this experiment.

That the hemotoxin was removed by a specific combination with the red cells was proved by washing the centrifuged red cells in cold salt solution and resuspending them in warm salt solution. Hemolysis of the red cells took place promptly.

Very recently, by a slightly different method, Neill and Fleming (11) have also been able to completely remove the hemotoxin from pneumococcus extracts by adsorption with red cells.

Antigenic Properties of the Necrosis-Producing Principle.

Three rabbits were injected intracutaneously at weekly intervals with 1 cc. doses of sterile necrotizing filtrates from *Pneumococcus* I. Six injections were given

TABLE I.
Action of Hemotoxin-Free Autolysate.

Autolysate	Hemotoxic action		Necrotizing action	
	Before adsorption	After adsorption	Before adsorption	After adsorption
0.1 cc.	++++	0	++++	++++
0.02 cc.	++++	0	+	+

in all. The last two inoculations caused no reaction at the sites of injection. The rabbits were bled 10 days after the last inoculation and their serums titrated for the presence of neutralizing antibodies.

These tests were carried out as follows: 0.9 cc. of a well chilled necrotizing filtrate was placed in each of three narrow test-tubes. To the first tube was added 0.1 cc. of the antiserum; to the second tube 0.1 cc. of normal rabbit serum; and to the third the same amount of broth. The contents of the tubes were well mixed, and a heavy vaseline seal was then added to each tube. After standing at room temperature for 1 hour, the tubes were again chilled, the vaseline seals removed, and the preparations in amounts of 0.1 cc. were injected intracutaneously into a small guinea pig. The results are summarized in Table II.

Numerous similar experiments have been made, and it has been found that our immune serum in dilution of 1-10 neutralized the necrotizing poison, whereas normal rabbit serum in the same dilution had no detoxicating effect. The immune serum diluted 1-20 only par-

tially neutralized the toxin. Occasionally slight inactivation occurred with normal serums diluted 1:5.

These experiments seem to show that the immunization of rabbits with necrotizing filtrates causes the production of neutralizing antibodies. Interpretation of these results, however, is complicated by the fact that considerable cloudiness develops in the mixtures of immune serum and toxin, probably due to the action of the antiprotein precipitins, which the antisera contain, on the pneumococcus protein which is present in the filtrates. From these experiments alone, it would be impossible to say whether the neutralization of the necrotizing poison by this immune serum is really a neutralization, or whether the active substance is merely carried down with the precipitated particles of protein. The same criticism applies to our experiments with *Pneumococcus* II autolysates as described below.

TABLE II.

Dilution of normal serum	Reaction on guinea pig	Dilution of Type I serum	Reaction on guinea pig
1-5	++	1-5	0
1-10	+++	1-10	0
1-20	+++	1-20	0

Is the Necrosis-Producing Principle Type-Specific or Species-Specific?

It is of considerable interest to know if the necrotizing autolysates prepared from other types of pneumococci can be neutralized by the serums produced against our preparations from *Pneumococcus* I. To investigate this point, we tested the neutralizing action of our *Pneumococcus* I antiserum against the necrotizing autolysates prepared from a *Pneumococcus* II strain which was isolated in October, 1927, from the blood of a patient with pneumonia at the Presbyterian Hospital. It was found that the Type I antinecrotizing serums in dilutions of 1-10 completely neutralized the Type II poison.

As far as they go, these experiments appear to indicate that the necrotizing principles of *Pneumococcus* I or II are antigenically similar and are probably, therefore, like the pneumococcus hemotoxin, species- and not type-specific.

CONCLUSIONS.

1. Certain pneumococcus autolysates produce necrosis when injected into the skin of guinea pigs.

2. The necrosis-producing principle can be filtered through a Berkefeld N filter, is extremely thermolabile, and is very sensitive to oxidation.

3. The necrotizing poison can be separated from the pneumococcus hemotoxin by adsorption with red cells. This removes the hemotoxin and leaves the necrosis-producing principle unaffected.

4. The necrotizing substances obtained from Pneumococcus Types I and II are neutralized by the antiserum prepared with Pneumococcus I.

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CALCIUM AND INORGANIC PHOSPHORUS IN THE BLOOD OF RABBITS.

I. RESULTS OF REPEATED AND PROLONGED OBSERVATIONS ON NORMAL RABBITS.

By WADE H. BROWN, M.D.

WITH THE COLLABORATION OF MARION HOWARD.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

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A study of calcium and inorganic phosphorus in the blood of rabbits was undertaken by us as a phase of a more comprehensive investigation of the problem of constitutional factors in relation to the occurrence and course of disease, with especial reference to the influence of environmental conditions (1). In extending this investigation to chemical constituents of the blood, it seemed desirable to begin with substances which are supposed to maintain some kind of equilibrium and for which quantitative methods have been developed that would permit of a systematic study in large series of animals or over long periods of time.

Calcium and inorganic phosphorus appeared to meet these fundamental requirements. Moreover, the work of Grant and Gates (2) indicated that the amounts of these substances in the blood of rabbits are subject to spontaneous (seasonal) variation, and hence, that they may be affected by environmental conditions—a supposition that is also supported by recent studies of rickets. There was the additional possibility that a relation might be found between the variations in calcium and inorganic phosphorus and the susceptibility of animals to disease, since organs concerned in regulating the concentration of these substances in the blood frequently show marked changes in the reaction to disease. This is especially true of the two diseases (syphilis and malignant disease) which have been used as the basis of our study of the influence of constitutional factors.

The experiments that have been carried out are divisible into two

groups: first, those dealing with conditions that obtain in normal rabbits, and, second, those concerned with animals inoculated with *Treponema pallidum* or a malignant tumor. The results obtained for normal animals will be reported first. The present paper will be limited to a consideration of mean normal values, and deviations from such values for calcium and inorganic phosphorus in the blood of rabbits as determined by repeated examinations of 4 groups of animals over periods of from 16 to 32 weeks. In subsequent papers, these basic results will be compared with other results from the point of view of the various factors inherent in the animal material or of an environmental (experimental) nature which may affect the calcium and inorganic phosphorus content of the blood in a given case.

Material and Methods.

There were several important considerations which governed the conduct of these experiments. In the first place, an effort was made to obtain a fair sample of the animal material available for experimental purposes, and the animals were caged and cared for in the usual manner. In the second place, the period of observation and the interval at which blood examinations were made were determined with reference to other experiments, while groups of animals were distributed so as to cover as long a period of time as possible.

The results reported below are based on examinations of 4 groups of male rabbits, most of which were 6 to 8 months old at the beginning of the experiments; a few were older, and a few others may have been a month or 6 weeks younger. The period covered by the investigation was from October 8, 1926, to July 1, 1927, inclusive. While under observation, the animals were caged separately and kept in well lighted, well ventilated rooms which were heated during the colder weather. The diet consisted of hay, oats and cabbage.

Group I.—10 rabbits, 5 white and 5 black. Between October 8, 1926, and May 18, 1927, the blood of these animals was examined at weekly or biweekly intervals.

Group II.—5 rabbits, gray, brown or black. These animals came from the same stocks as those of Group I and were placed under observation at the same time, but no blood examinations were made until December 1, 1926, or until they had been caged and under observation for approximately 2 months. Blood analyses on these animals were made at somewhat irregular intervals, but, for the most part, they paralleled those of Group I. The period covered was from December 1, 1926, to June 8, 1927.

Group III.—10 rabbits, grays and browns. These animals were slightly younger than those of Groups I and II. Biweekly examinations were made from January 14 to July 1, 1927.

Group IV.—11 rabbits, browns and grays. Most of these animals were still

younger than those of Group III. From March 11 to May 13 examinations were made at weekly intervals and biweekly from May 13 to July 1, 1927.

The observations made on the 4 groups of rabbits may be summarized as follows:

Group	Observation period	Group determinations	Individual determinations
I	Oct. 8 to May 18	22	216
II	Dec. 1 to June 8	17	85
III	Jan. 14 to July 1	15	143
IV	Mar. 11 to July 1	15	153
Combined	Oct. 8 to July 1	69	597

Determinations of calcium and inorganic phosphorus were made on the blood serum according to the methods of Kramer and Tisdall (3) and of Tisdall (4) respectively.

At first, the procedure followed was somewhat irregular with respect to the disposition of the blood after it was drawn and the interval of time between bleeding and analysis. In some instances, the blood was permitted to stand at room temperature for several hours, or until there was a well defined separation of serum and clot, and then transferred to the ice box until the following morning when the analyses were made. It thus happened that an interval of 12 to 18 hours elapsed between bleeding and analysis.

After the first few weeks, however, a uniform procedure was adopted. On the day preceding examination, animals were fed as usual in the afternoon and were bled early the following morning before they had received any additional food. 8 cc. of blood was drawn from a marginal ear vein into a graduated centrifuge tube and permitted to clot at room temperature. The clot was separated from the wall of the tube and as soon as sufficient serum was expressed, the blood was centrifuged and analysis commenced at once. The time between the bleeding and analysis was thus reduced to 1 to 2 hours, and the use of the ice box was eliminated.

In order to expedite the work, and as a precautionary measure in the case of syphilitic animals, the serum was measured with a 1 cc. tuberculin syringe graduated to 0.01 cc. instead of a pipette. The syringes were fitted with 18-20 gauge needles and their accuracy was carefully controlled. As far as possible, the work was so arranged that a given procedure was always performed by the same person; all titrations and all colorimetric readings were made by one person (Howard).

The results given represent determinations of calcium and phosphorus on the same samples of blood. Numerous determinations were made in duplicate to control the experimental error in the methods as carried out. In such cases, the mean value of the determinations was recorded and used as a single determination in the results to be reported. The mean error for duplicate determinations of

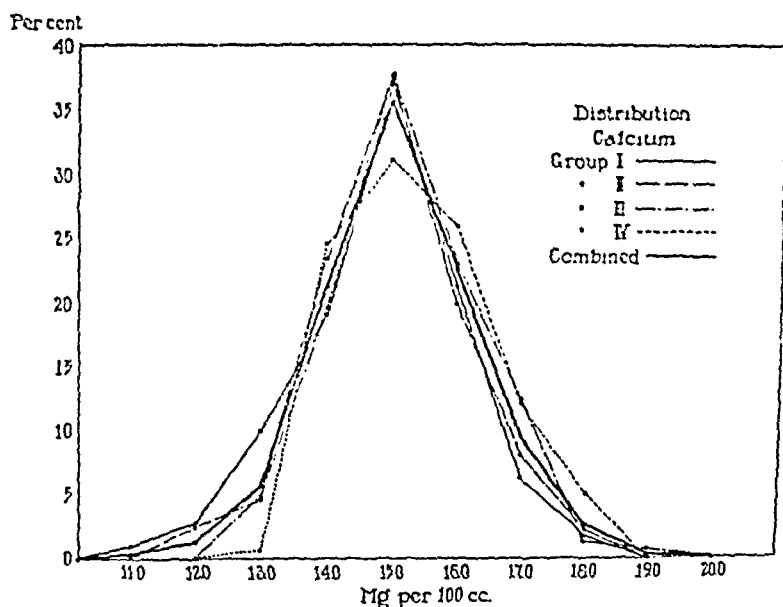
calcium and inorganic phosphorus was approximately 0.2 mg. per 100 cc. of serum, or about 1.25 per cent and 4.50 per cent of the respective mean values.

In addition to the primary values for calcium and inorganic phosphorus, we have computed values for the sum and product of the two substances, the ratio of calcium to inorganic phosphorus, and of the product to the sum, and for the sum of these two ratios.

The results are presented in the form of tabulated summaries (Tables I to XV) and a series of text-figures (Text-figs. 1 to 7). Values are given in Tables I to XV for individual groups of animals and for the 4 groups combined. The combined values do not represent means, but are obtained by actual combination of all observations. It will be noted that a few of the higher values given in the distribution tables are not recorded in the text-figures.

RESULTS.

As the purpose of this paper is to give a general idea of the calcium and inorganic phosphorus content of rabbits' blood, under certain prescribed conditions, it seems unnecessary to record the details of individual examinations. The results are reported, therefore, in the form of tabulated summaries (Tables I to XV) supplemented by a series of graphs (Text-figs. 1 to 7).



TEXT-FIG. 1.

TABLE I.

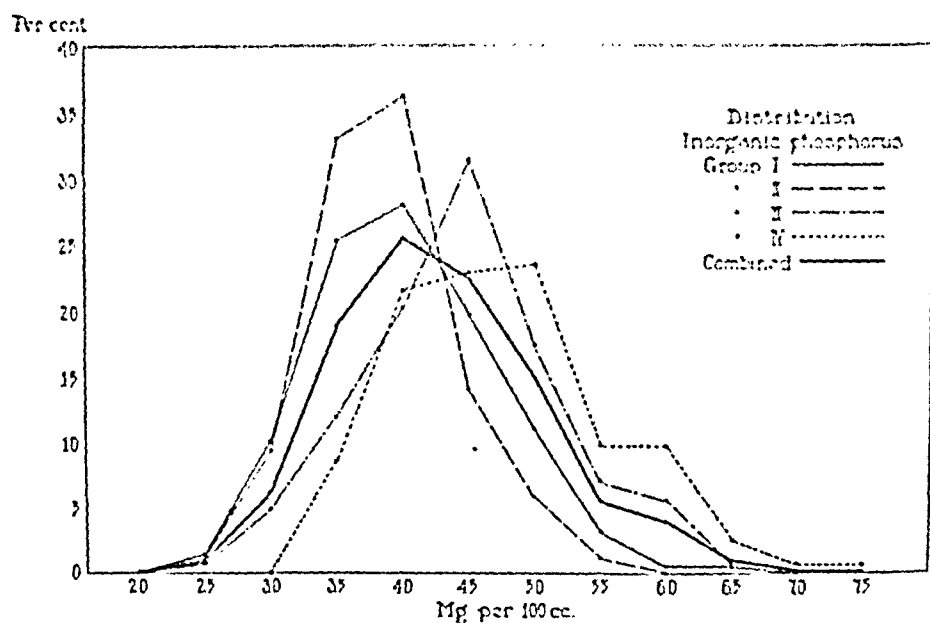
Calcium.

Group	Number of observations	Mean	Minimum	Maximum	Standard deviation	Coefficient of variation
		<i>mg. per 100 cc.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>per cent</i>
I	219	15.3±0.06	11.1	21.2	1.30	8.50
II	85	15.5±0.10	12.6	22.8	1.38	8.90
III	143	15.7±0.07	13.4	19.7	1.17	7.45
IV	154	15.8±0.06	13.8	18.9	1.12	7.09
Combined	601	15.6±0.03	11.1	22.8	1.25	8.01

TABLE II.

Distribution of Values for Calcium.

Mg. per 100 cc.	Group I	Group II	Group III	Group IV	Combined
11.0-11.4	1				1
11.5-11.9	1				1
12.0-12.4					
12.5-12.9	6	2			8
13.0-13.4	10	1	2		13
13.5-13.9	12	3	5	1	21
14.0-14.4	18	7	11	17	53
14.5-14.9	24	13	17	21	75
15.0-15.4	40	25	32	28	125
15.5-15.9	41	7	22	20	90
16.0-16.4	35	9	21	25	90
16.5-16.9	12	8	12	15	47
17.0-17.4	10	5	7	12	34
17.5-17.9	4	2	11	7	24
18.0-18.4	2	1		6	9
18.5-18.9	2	1	2	2	7
19.0-19.4					
19.5-19.9			1		1
20.0-20.4					
20.5-20.9					
21.0-21.4	1				1
21.5-21.9					
22.0-22.4					
22.5-22.9		1			1
Observations	219	85	143	154	601



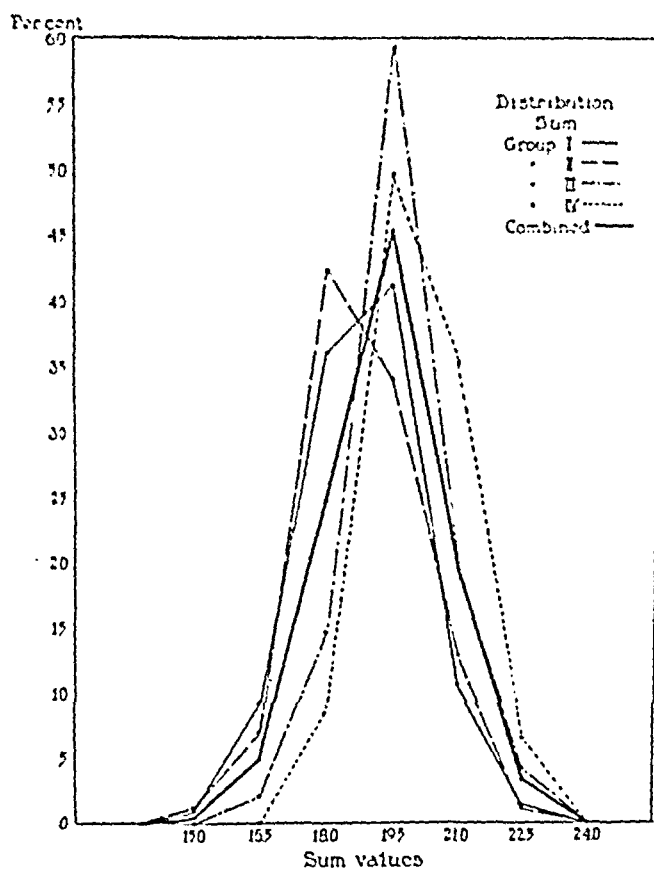
TEXT-FIG. 2.

TABLE III.
Inorganic Phosphorus.

Group	Number of observations	Mean	Minimum	Maximum	Standard deviation	Coefficient of variation
		<i>mg. per 100 cc.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>per cent</i>
I	216	4.25±0.03	2.60	6.74	0.68	16.00
II	85	4.11±0.04	2.95	5.77	0.51	12.41
III	143	4.67±0.04	2.64	6.76	0.75	16.06
IV	153	4.96±0.04	3.59	7.50	0.80	16.13
Combined	597	4.51±0.02	2.60	7.50	0.78	17.29

TABLE IV.
Distribution of Values for Inorganic Phosphorus.

Mg. per 100 cc.	Group I	Group II	Group III	Group IV	Combined
2.50-2.74	2		1		3
2.75-2.99	1	1			2
3.00-3.24	7		2		9
3.25-3.49	15	8	5		28
3.50-3.74	18	13	10	5	46
3.75-3.99	37	15	7	8	67
4.00-4.24	33	17	11	17	78
4.25-4.49	27	13	18	16	74
4.50-4.74	27	9	29	25	90
4.75-4.99	16	3	16	10	45
5.00-5.24	15	4	13	22	54
5.25-5.49	9	1	12	14	36
5.50-5.74	4		6	11	21
5.75-5.99	3	1	4	4	12
6.00-6.24			8	8	16
6.25-6.49	1			7	8
6.50-6.74	1			3	4
6.75-6.99			1	1	2
7.00-7.24				1	1
7.25-7.49					
7.50-7.74				1	1
Observations	216	85	143	153	597



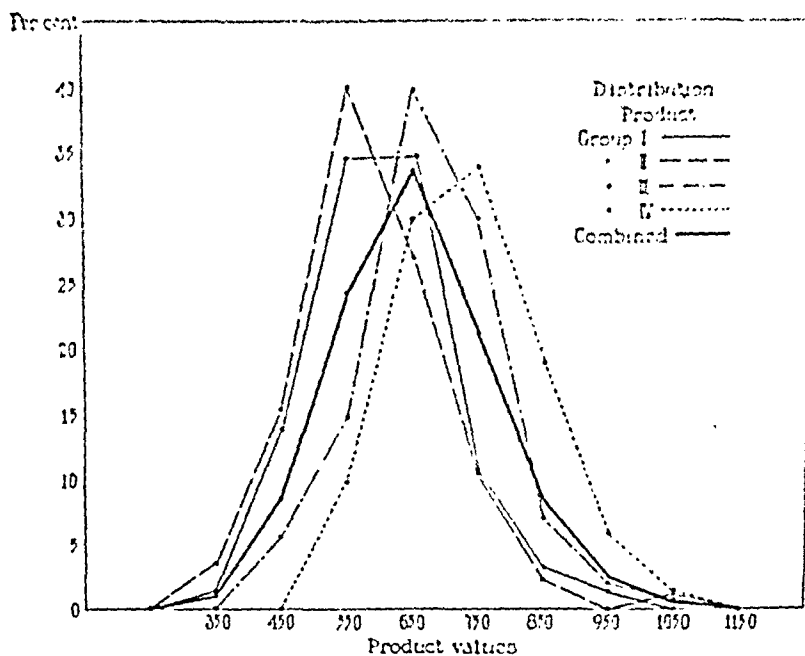
TEXT-FIG. 3.

TABLE V.
Sum of Calcium and Inorganic Phosphorus.

Group	Number of observations	Mean	Minimum	Maximum	Standard deviation	Coefficient of variation <i>per cent</i>
I	216	19.6 ± 0.06	15.51	25.81	1.31	6.68
II	85	19.7 ± 0.11	16.40	27.50	1.53	7.77
III	143	20.4 ± 0.06	17.50	23.90	1.11	5.44
IV	153	20.8 ± 0.06	18.30	23.60	1.01	4.86
Combined	597	20.1 ± 0.04	15.51	27.50	1.29	6.42

TABLE VI.
Distribution of Values for the Sum of Calcium and Inorganic Phosphorus.

Sum values	Group I	Group II	Group III	Group IV	Combined
15.5-15.9	1				1
16.0-16.4	1	1			2
16.5-16.9	2	2			4
17.0-17.4	4	1			5
17.5-17.9	14	3	3		20
18.0-18.4	14	7		2	23
18.5-18.9	24	11	5	5	45
19.0-19.4	40	18	16	6	80
19.5-19.9	34	8	31	15	88
20.0-20.4	32	13	29	29	103
20.5-20.9	23	8	25	32	88
21.0-21.4	11	4	12	23	50
21.5-21.9	7	6	11	21	45
22.0-22.4	5	1	5	10	21
22.5-22.9	3	1	2	7	13
23.0-23.4			1	2	3
23.5-23.9			3	1	4
24.0-24.4					
25.5-25.9	1				1
27.5-27.9		1			1
Observations	216	85	143	153	597



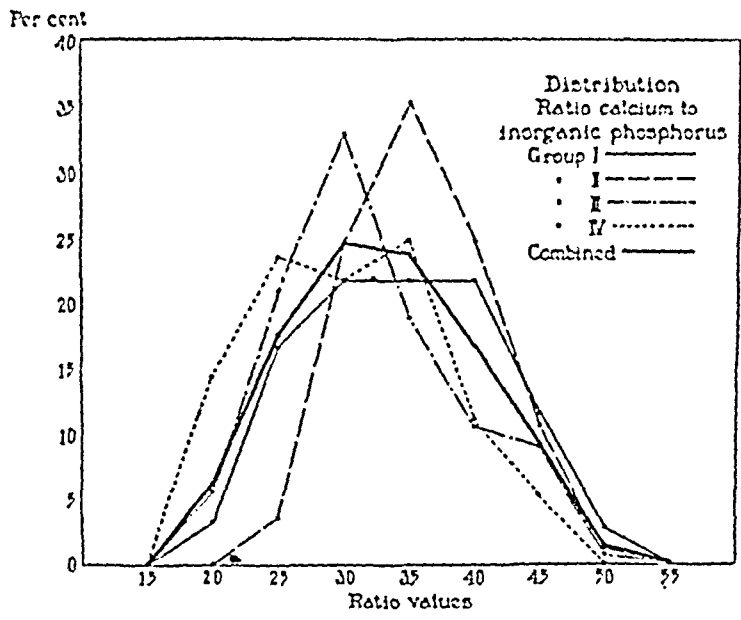
TEXT-FIG. 4.

TABLE VII.
Product of Calcium and Inorganic Phosphorus.

Group	Number of observations	Mean	Minimum	Maximum	Standard deviation	Coefficient of variation
						<i>per cent</i>
I	216	65.3±0.5	40.3	99.8	10.4	15.93
II	85	64.0±0.8	43.5	108.1	10.6	16.56
III	143	73.0±0.6	45.2	107.9	10.8	14.79
IV	153	78.1±0.6	55.9	113.9	10.8	13.83
Combined	597	70.2±0.3	40.3	113.9	12.0	17.09

TABLE VIII.
Distribution of Values for the Product of Calcium and Inorganic Phosphorus.

Product values	Group I	Group II	Group III	Group IV	Combined
40- 44	3	3			6
45- 49	10		2		12
50- 54	20	13	6		39
55- 59	38	19	10	4	71
60- 64	37	15	11	11	74
65- 69	44	15	22	20	101
70- 74	31	8	35	26	100
75- 79	15	6	22	30	73
80- 84	8	3	21	22	54
85- 89	5	2	7	18	32
90- 94	2		3	11	16
95- 99	3		1	7	11
100-104			2	2	4
105-109		1	1	1	3
110-114				1	1
Observations	216	85	143	153	597



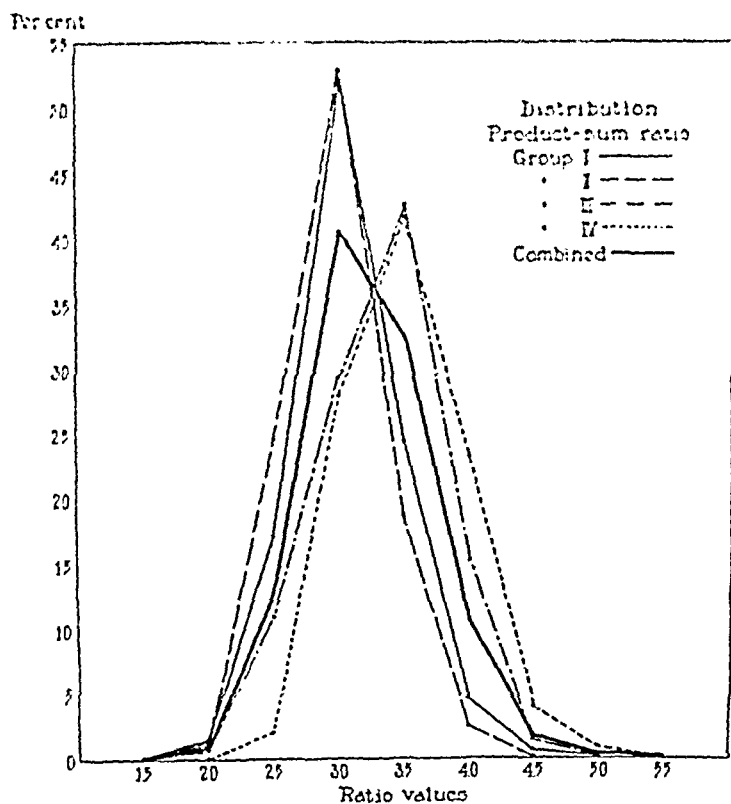
TEXT-FIG. 5.

TABLE IX.
Ratio of Calcium to Inorganic Phosphorus.

Group	Number of observations	Mean	Minimum	Maximum	Standard deviation	Coefficient of variation
						<i>per cent</i>
I	216	3.70 ± 0.03	2.16	6.37	0.73	19.73
II	85	3.83 ± 0.04	2.66	5.00	0.53	13.84
III	143	3.47 ± 0.04	2.19	6.48	0.74	21.33
IV	153	3.30 ± 0.04	2.03	4.71	0.65	19.70
Combined	597	3.57 ± 0.02	2.03	6.48	0.72	20.17

TABLE X.
Distribution of Values for the Ratio of Calcium to Inorganic Phosphorus.

Ratio values	Group I	Group II	Group III	Group IV	Combined
2.00-2.24	2		1	6	9
2.25-2.49	5		7	16	28
2.50-2.74	15	1	16	11	43
2.75-2.99	21	2	14	25	62
3.00-3.24	23	7	21	23	74
3.25-3.49	24	14	26	9	73
3.50-3.74	22	11	18	23	74
3.75-3.99	25	19	9	15	68
4.00-4.24	26	13	7	16	62
4.25-4.49	21	8	8	1	38
4.50-4.74	17	3	8	8	36
4.75-4.99	8	6	5		19
5.00-5.24	3	1			4
5.25-5.49	3		1		4
5.50-5.74			1		1
5.75-5.99					
6.00-6.24					
6.25-6.49	1		1		2
Observations	216	85	143	153	597



TEXT-FIG. 6.

TABLE XI.

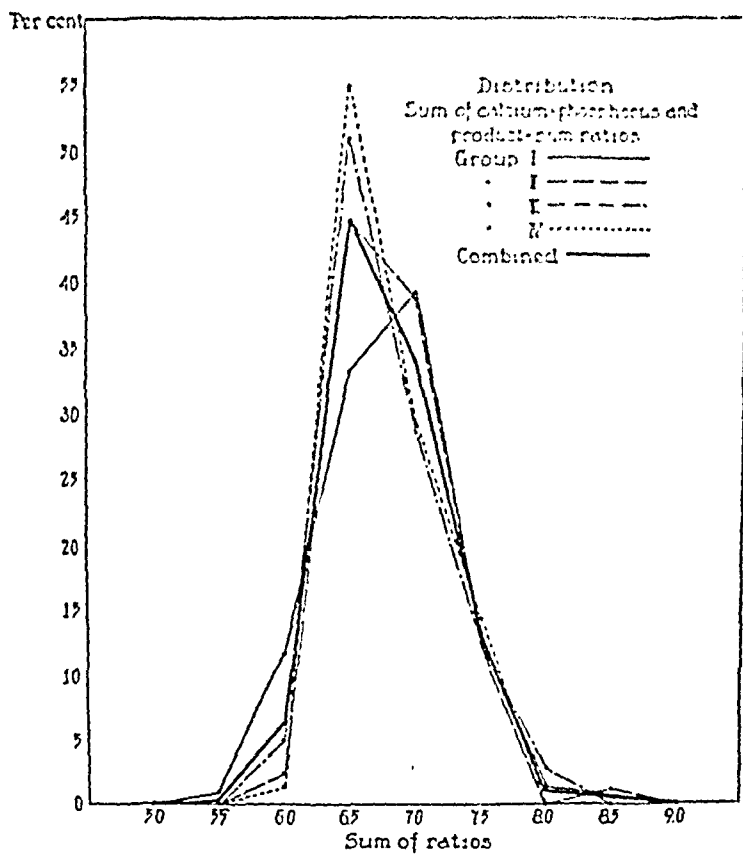
Ratio of Product to the Sum of Calcium and Inorganic Phosphorus.

Group	Number of observations	Mean	Minimum	Maximum	Standard deviation	Coefficient of variation
						<i>per cent</i>
I	216	3.32 ± 0.02	2.25	4.63	0.39	11.75
II	85	3.24 ± 0.02	2.46	4.19	0.33	10.19
III	143	3.58 ± 0.02	2.29	4.68	0.40	11.17
IV	153	3.75 ± 0.02	2.96	5.02	0.42	11.20
Combined	597	3.47 ± 0.01	2.25	5.02	0.44	12.68

TABLE XII.

Distribution of Values for the Ratio of the Product to the Sum of Calcium and Inorganic Phosphorus.

Ratio values	Group I	Group II	Group III	Group IV	Combined
2.25-2.49	3	1	1		5
2.50-2.74	13	4	3		20
2.75-2.99	24	17	12	3	56
3.00-3.24	54	27	11	16	108
3.25-3.49	58	18	31	27	134
3.50-3.74	31	11	40	33	115
3.75-3.99	22	5	21	31	79
4.00-4.24	8	2	16	21	47
4.25-4.49	2		6	15	23
4.50-4.74	1		2	5	8
4.75-4.99				1	1
5.00-5.24				1	1
Observations	216	85	143	153	597



TEXT-FIG. 7.

TABLE XIII.

Sum of the Ratios of Calcium to Inorganic Phosphorus and of the Product to the Sum.

Group	Number of observations	Mean	Minimum	Maximum	Standard deviation	Coefficient of variation
						<i>per cent</i>
I	216	7.02 ± 0.02	5.68	8.62	0.45	6.41
II	85	7.07 ± 0.03	6.11	8.74	0.41	5.80
III	143	7.05 ± 0.02	6.32	8.77	0.41	5.82
IV	153	7.05 ± 0.02	6.49	7.85	0.33	4.68
Combined	597	7.04 ± 0.01	5.68	8.77	0.40	5.68

TABLE XIV.

Distribution of Values for the Sum of the Ratios of Calcium to Inorganic Phosphorus and of the Product to the Sum of the Two Substances.

Sum values	Group I	Group II	Group III	Group IV	Combined
5.50-5.74	1				1
5.75-5.99	1				1
6.00-6.24	10	2			12
6.25-6.49	15		7	2	24
6.50-6.74	32	12	29	32	105
6.75-6.99	40	26	44	52	162
7.00-7.24	54	20	21	25	121
7.25-7.49	31	13	20	20	83
7.50-7.74	20	7	15	18	60
7.75-7.99	8	4	3	4	19
8.00-8.24	2		2		4
8.25-8.49	1		1		2
8.50-8.74	1	1			2
8.75-8.99			1		1
Observations	216	85	143	153	597

TABLE XV.
Summary of Results.

Group	Number of animals	Number of observations	Observation period wks.	Calcium mg. per 100 cc.	Inorganic phosphorus mg. per 100 cc.	Sum of calcium and phosphorus	Product of calcium and phosphorus	Ratio of calcium to phosphorus	Ratio of product to sum	Sum of two ratios
I	10	216	32	15.3	4.25	19.6	65.3	3.70	3.32	7.02
II	5	85	27	15.5	4.11	19.7	64.0	3.83	3.24	7.07
III	10	143	25	15.7	4.67	20.4	73.0	3.47	3.58	7.05
IV	11	153	16	15.8	4.96	20.8	78.1	3.30	3.75	7.05
Combined	36	597	37.5	15.6	4.51	20.1	70.2	3.57	3.47	7.04

DISCUSSION AND CONCLUSIONS.

It will be seen at once that the mean values obtained for calcium are higher than most of those recorded in the literature, and that the values for inorganic phosphorus are perhaps lower. It is well to bear in mind, however, that the significance that may be attached to any series of determinations of calcium and inorganic phosphorus in the blood of animals depends largely upon the conditions under which the determinations are made. As is well known, there are many factors that may affect the values obtained, including inherent differences in the animal material and the method of analysis used as well as the particular procedure employed in carrying out a given method. When all other conditions are uniform, irregularities in the handling of the blood after it is drawn will give rise to surprisingly large differences in the results for both calcium and inorganic phosphorus, as permitting blood to stand tends to decrease calcium values and to increase those for inorganic phosphorus.

It seems desirable, therefore, to emphasize the fact that the results recorded above are to be viewed as results obtained under certain definitely prescribed conditions which differ in several important respects from those governing determinations made by other workers in this field. Moreover, it is to be noted that the conditions varied to some extent with each of the 4 groups of animals comprising this

series. For example, there was a small but definite age difference. The animals of Groups I and II were older and more mature than those of Groups III and IV at the beginning of the experiments, and this initial difference was increased by the extension of the experiments on Groups I and II over a longer period of time, so that the observations made on these animals not only included data for a more advanced age, but represented a mean age considerably above that of the observations made on the animals of Groups III and IV. There was a similar difference of experimental conditions between Groups III and IV, while the observations on Group II differed from those on Group I in that no blood analyses were made on the animals of Group II for 2 months after they were placed under observation.

These particular features of the experiments are mentioned because an examination of the text-figures will show that a line of cleavage between Groups I and II on the one hand, and III and IV on the other, is traceable through all of the distribution curves and to some extent in the tabulated results. With the combined values as the axis of distribution, Groups I and II invariably hang together, or swing to one side, while Groups III and IV swing to the other. Moreover, the extreme positions are usually represented by Groups II and IV.

Whether these peculiarities of the results are in reality attributable to the conditions mentioned or to some other cause, such as the length of cage life (2), or the particular period covered by the observations, the suggested relation is sufficient to indicate the extent to which even slight differences in experimental conditions may affect the results obtained for blood calcium and inorganic phosphorus.

The values obtained for calcium may be regarded as showing a fairly close agreement (Tables I and II and Text-fig. 1). The extreme difference between the means for the 4 groups of animals is only 0.5 mg. or approximately 3.00 per cent of the mean for the combined groups. Still, the small absolute difference between the means for Groups I and IV is nearly 6 times its probable error and, hence, cannot be disregarded.

The most important feature of these results is, however, the range of normal variation. The distribution curves (Text-fig. 1) show a remarkably close agreement in the frequency with which values of a

given magnitude occurred and an unusually symmetrical distribution of all values. The coefficients of variation are comparatively small (7.09 to 8.9 per cent), but values anywhere between 14.0 and 16.0 mg. of calcium per 100 cc. of serum occurred with great frequency, while figures as low as 13.5 or as high as 17.5 mg. (Table II) were by no means rare; and the extreme limits of observation indicate a potential difference in the calcium content of the blood of normal rabbits of as much as 100 per cent.

Inorganic phosphorus was found to be subject to much wider variation than calcium (Tables III and IV and Text-fig. 2). The coefficient of variation is approximately twice that for calcium (17.29 and 8.01 respectively), while the group means for phosphorus show a difference of 0.85 mg. per 100 cc. of serum. This difference is small in absolute value, but is nearly 20.0 per cent of the mean for all groups and is 15 times its probable error. It is safe to assume, therefore, that the values obtained indicate an actual difference in the inorganic phosphorus in the blood of the several groups of animals.

This conclusion is borne out by the distribution frequencies (Table IV and Text-fig. 2) which show that the values obtained for Groups I and II lie at a distinctly lower level than those for Groups III and IV; the difference between modal classes is, in fact, of the same order as that shown by the means.

The limits of probable variation as determined by the standard deviation of the combined results are 3.73 and 5.29 mg. per 100 cc. of serum, but one-third of all values lie outside of these limits, while the extreme limits of normal are sufficiently wide to include values that may differ by as much as 200.0 or even 300.0 per cent.

From the values obtained for calcium and inorganic phosphorus, the relation existing between the two substances may be measured in a number of ways. The ratio of the calcium to the phosphorus and the product of the amounts of the two substances have received the greatest attention. In addition to these values, we have computed values for the sum and for the ratio of the product to the sum, and also for the sum of the calcium-phosphorus ratio and the product-sum ratio.

The value for the sum of the calcium and inorganic phosphorus in the serum is determined largely by the calcium, but as it is also affected by the phosphorus, one might expect that the constancy of the value

as compared with that of calcium would be diminished unless the variations in the two substances were so related as to neutralize each other. As is well known, there is an apparent tendency in this direction and in these experiments it was found that on the whole the values for the sum showed less variation (coefficients 6.42 and 8.01 per cent) and were more uniformly distributed than those for calcium (Tables V and VI and Text-fig. 3). It is true that differences between groups were distinctly greater than in the case of calcium, but the agreement is sufficiently close to give evidence of a tendency to the maintenance of an inverse relation between serum calcium and inorganic phosphorus.

Values for the product of calcium and inorganic phosphorus emphasize the phosphorus factor rather than the calcium, reversing the conditions that obtain in the case of the sum. A consideration of the product values given in Tables VII and VIII and Text-fig. 4 show that, while the order of variation is essentially the same as that of inorganic phosphorus (coefficients 17.09 and 17.29 per cent respectively), the distribution of values is more uniform. This may be attributable to the occurrence of coordinate variations in calcium and inorganic phosphorus.

The situation presented by the values obtained for the ratio of calcium to inorganic phosphorus is somewhat surprising in that the ratio between the two substances proves to be less constant than the absolute amounts of either substance (Tables IX and X and Text-fig. 5). There are considerable differences between the standard values for individual groups of animals, and the distribution frequencies are inclined to be irregular. Moreover, all groups show a large standard deviation and correspondingly high coefficients of variation, but combining the results for the 4 groups of animals gives a fairly uniform and symmetrical distribution, a striking feature of which is the high frequency with which values occur over the entire range of standard variation, that is, from ratios of 2.85 to 4.29.

It thus appears that, despite the evidence of a tendency to the observance of an inverse relation between the calcium and inorganic phosphorus in the blood, the ratio of one substance to the other is by no means constant.

By using the product and the sum as a basis of expressing the relation between calcium and inorganic phosphorus, the form of the relation is

reversed, that is, an increase in calcium or a reduction in phosphorus diminishes the ratio value, whereas under the same circumstances, the direct ratio of calcium to phosphorus is increased. Objection may be made to this method of treatment, but the fact remains that by compounding the terms of the ratio in this manner, some interesting points are brought out (Tables XI and XII and Text-fig. 6).

In the first place, the mean values for the two ratios are of a comparable order, but the values obtained for the product-sum ratio of different groups of animals show a closer agreement than those for the calcium-phosphorus ratio. Moreover, the distribution of values is comparatively uniform throughout, while the variability of individual groups and of the series as a whole is less than two-thirds of that found for the calcium-phosphorus ratio.

Finally, known differences in the experimental conditions represented by the 4 groups of animals are expressed with remarkable clarity (Text-fig. 6) by this treatment of ratio values which suggests that the method may be used to advantage in the analysis of results of this kind.

By comparing the group values for the calcium-phosphorus and product-sum ratios, it will be seen that they are not only of a comparable order of magnitude (3.57 and 3.47 for the combined groups), but that a high value of one tends to be associated with a correspondingly low value of the other, so that the mean of the two ratios remains fairly constant at about 3.50, or their sum is approximately 7.00 (Tables XIII and XIV and Text-fig. 7).

The constancy of this relation does not hold for individual observations or for all classes of animals or experimental conditions. With grouped material, the absolute value of the sum or mean of the two ratios may increase or decrease, just as other values do, but tends to preserve a greater degree of constancy under a given set of conditions and appears to be less affected by slight changes in experimental conditions or by experimental errors. It is not certain whether the constancy of this value is due entirely to weighting, or the compounding of terms, or is indicative of the maintenance of a relation between calcium and inorganic phosphorus which is expressed in the maintenance of an equilibrium between the two ratios.

The values for calcium and inorganic phosphorus, as recorded above,

may be regarded as a cross-section of results obtained from a fair sample of laboratory material, and in this sense, may be used as standards of comparison in estimating the probable significance of other determinations. It has been clearly shown, however, that even slight differences in experimental conditions are apt to be reflected in the results obtained. It is evident, therefore, that as experimental conditions are changed, corresponding differences in results may be expected.

It has also been shown that neither the calcium nor the inorganic phosphorus of rabbits' blood can be regarded as constant. As is well known, inorganic phosphorus is the more labile of the two substances and is subject to much greater variation than calcium, but the upper and lower limits of both are far apart. In fact, the range of so called normal variation, or even the range of high frequency variation, is in all probability sufficiently wide to include many variations that occur under pathological as well as physiological conditions.

SUMMARY.

Determinations of calcium and inorganic phosphorus were made on the blood of 4 groups of normal rabbits at intervals of 1 to 2 weeks over periods of 4 to 8 months. From the data thus obtained, values were calculated for calcium and inorganic phosphorus, and for various relations between the two substances.

It was found that both calcium and inorganic phosphorus showed wide ranges of variation with a tendency to vary in an inverse direction. Still, the relation between the two substances, as expressed by a direct ratio or by the product of the two substances, was less constant than the absolute amounts of either calcium or inorganic phosphorus. The ratio of the product to the sum of the two substances was more constant, and this ratio varied inversely with the ratio of calcium to inorganic phosphorus, so that the mean or the sum of the two values varied comparatively little under a given set of experimental conditions.

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CALCIUM AND INORGANIC PHOSPHORUS IN THE BLOOD OF RABBITS.

II. RESULTS OF SINGLE DETERMINATIONS ON NORMAL RABBITS FROM RECENTLY ACQUIRED STOCKS.

By WADE H. BROWN, M.D.

WITH THE COLLABORATION OF MARION HOWARD.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

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Results of a large series of determinations of calcium and inorganic phosphorus in the blood of normal rabbits were reported in a previous paper (1). These results were based on repeated examination of the blood of the same animals, continued over periods of 4 to 8 months. A parallel series of blood analyses was made on small groups of animals within a few days after they were received from the dealer, for the purpose of determining whether the calcium and inorganic phosphorus in the blood of rabbits, as they come to the laboratory from outside sources, differed in any way from that of rabbits living under the usual laboratory conditions. The results of this investigation are presented in the present paper in the form of a comparison of group means, variability and distribution frequencies.

Material and Methods.

The results to be reported are based on determinations of calcium and inorganic phosphorus in one sample of blood from each of 111 male rabbits distributed as follows:

Oct. 8, 1926.....	10	rabbits
Nov. 17, 1926.....	10	"
Jan. 7-14, 1927.....	21	"
Feb. 4, 1927.....	11	"
Mar. 11, 1927.....	32	"
Apr. 29, 1927.....	10	"
May 25, 1927.....	9	"
June 8, 1927.....	8	"

The blood of the first group of animals and of some of those in the third group was not examined until they had been in the laboratory about 10 days; in other cases, the analyses were made between 1 and 3 days after the animals were received from the dealer.

These animals were comparable with those used for repeated determinations of calcium and inorganic phosphorus. In fact, the observations on the October group of this series were continued, constituting Group I of the series of repeated determinations already reported upon, while parts of the January and March animals formed Groups III and IV respectively of the first series.

Calcium and inorganic phosphorus were determined in accordance with the methods described in the previous paper (1).

The results of these determinations have been combined and analyzed as a single group in order to provide a basis for direct comparison with results obtained by repeated examination. For convenience, the series of repeated determinations will be designated as Series I, and the single determinations as Series II. A summary of results is given in Table I which, in addition to the values for Series II, contains the highest and lowest group values for Series I as well as the combined values for all groups of the series. As an aid to the interpretation of these results, the differences between mean values and the relation of these differences to their probable errors have also been tabulated (Table II). Tables III to V give the distribution frequencies for Series II with corresponding combined values for Series I. These relations are shown graphically in Text-figs. 1 to 7.

RESULTS.

The results of the investigation are summarized in Tables I to V and Text-figs. 1 to 7.

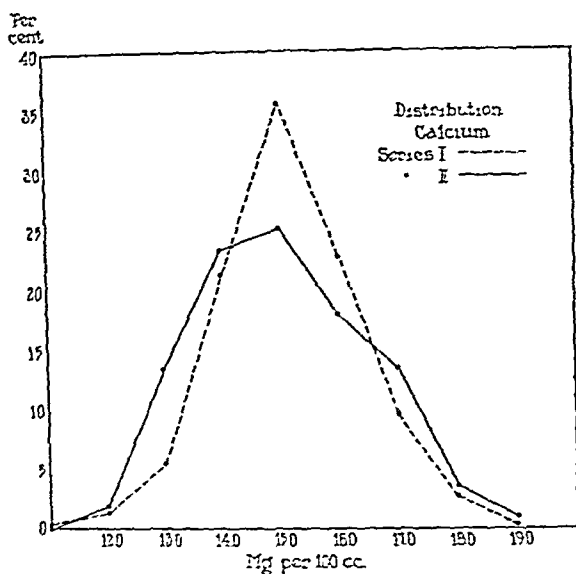
TABLE I.
*Values for Series of Single and Repeated Determinations of Calcium and
 Inorganic Phosphorus.*

	Mean	Standard deviation	Coefficient of variation
	<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>	<i>per cent</i>
Calcium			
Series I High	15.8±0.06	1.12	7.09
Low	15.3±0.06	1.30	8.50
Combined	15.6±0.03	1.25	8.01
Series II	15.4±0.09	1.42	9.20
Inorganic phosphorus			
Series I High	4.96±0.04	0.80	16.13
Low	4.11±0.04	0.51	12.41
Combined	4.51±0.02	0.78	17.29
Series II	5.40±0.06	0.91	16.85
Sum	Absolute values	Absolute values	
Series I High	20.8±0.06	1.01	4.86
Low	19.6±0.06	1.31	6.63
Combined	20.1±0.04	1.29	6.42
Series II	20.8±0.11	1.70	8.17
Product			
Series I High	78.1±0.6	10.8	13.83
Low	64.0±0.8	10.6	16.56
Combined	70.2±0.3	12.0	17.09
Series II	83.3±1.03	16.0	19.22
Calcium-phosphorus ratio			
Series I High	3.83±0.04	0.53	13.84
Low	3.30±0.04	0.65	19.70
Combined	3.57±0.02	0.72	20.17
Series II	2.95±0.04	0.57	19.32
Product-sum ratio			
Series I High	3.75±0.02	0.42	11.20
Low	3.24±0.02	0.33	10.19
Combined	3.47±0.01	0.44	12.68
Series II	3.95±0.03	0.51	12.91
Sum of two ratios			
Series I High	7.07±0.03	0.41	5.80
Low	7.02±0.02	0.45	6.41
Combined	7.04±0.01	0.40	5.68
Series II	6.91±0.02	0.37	5.35

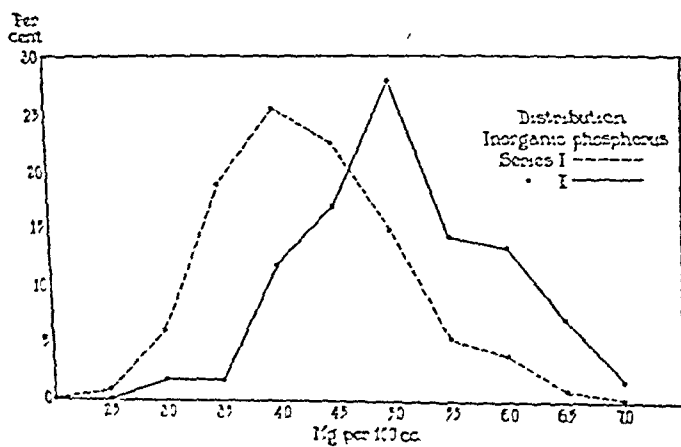
TABLE II.

Differences between Group Means as Compared with Their Probable Errors.

	Difference	Difference P.E.
Calcium		
1. Series I, highest and lowest values	0.5 mg.	6
2. Series I, combined value and Series II	0.2 "	2
3. Series I, combined and highest values	0.2 "	3
4. Series I, combined and lowest values	0.3 "	4
Phosphorus		
1. Series I, highest and lowest values	0.85 mg.	15
2. Series I, combined value and Series II	0.89 "	14
3. Series I, combined and highest values	0.45 "	10
4. Series I, combined and lowest values	0.40 "	9
Sum		
1. Series I, highest and lowest values	1.2	14
2. Series I, combined value and Series II	0.7	6
3. Series I, combined and highest values	0.7	10
4. Series I, combined and lowest values	0.5	7
Product		
1. Series I, highest and lowest values	14.1	14
2. Series I, combined value and Series II	15.1	9
3. Series I, combined and highest values	7.9	12
4. Series I, combined and lowest values	6.2	7
Ratio of calcium to phosphorus		
1. Series I, highest and lowest values	0.53	9
2. Series I, combined value and Series II	0.62	15
3. Series I, combined and highest values	0.26	6
4. Series I, combined and lowest values	0.27	6
Ratio of product to sum		
1. Series I, highest and lowest values	0.51	18
2. Series I, combined value and Series II	0.48	15
3. Series I, combined and highest values	0.28	13
4. Series I, combined and lowest values	0.23	11
Sum of two ratios		
1. Series I, highest and lowest values	0.05	1
2. Series I, combined value and Series II	0.13	6
3. Series I, combined and highest values	0.03	Less than P. E.
4. Series I, combined and lowest values	0.02	Less than P. E.



TEXT-FIG. 1.

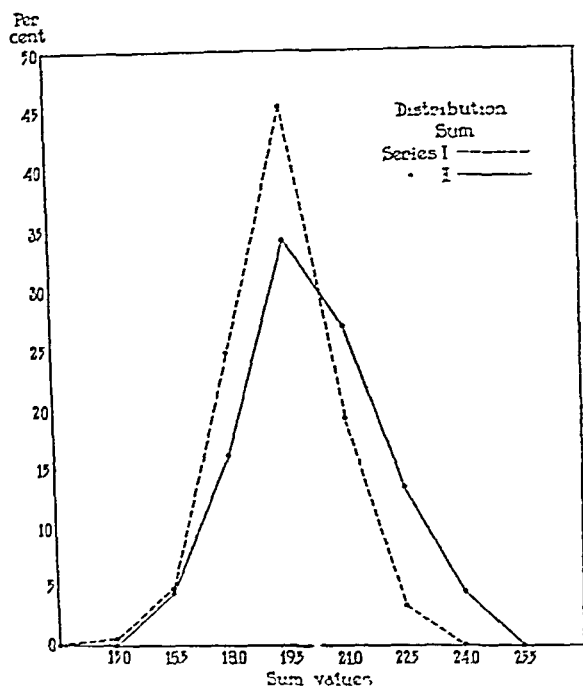


TEXT-FIG. 2.

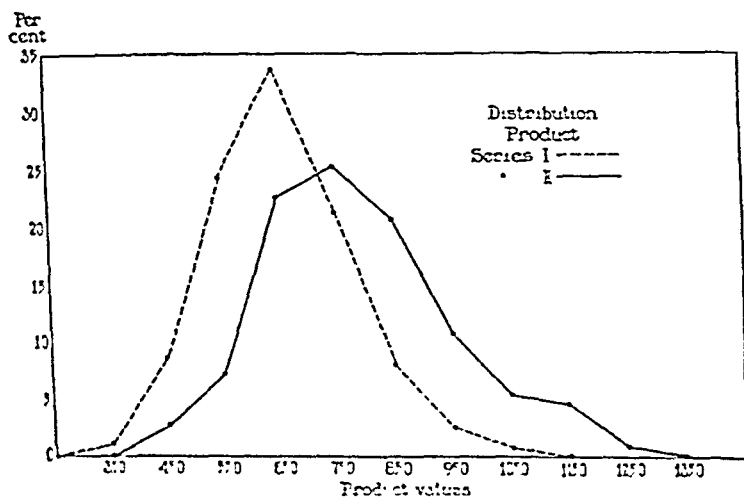
TABLE III.

Distribution of Values for Calcium and Inorganic Phosphorus.

Calcium	Series I	Series II	Inorganic phosphorus	Series I	Series II
<i>mg. per 100 cc.</i>	<i>per cent</i>	<i>per cent</i>	<i>mg. per 100 cc.</i>	<i>per cent</i>	<i>per cent</i>
11.0	0.34		2.00		
12.0	1.33	1.80	2.50	0.85	
13.0	5.66	13.52	3.00	6.18	1.80
14.0	21.32	23.42	3.50	18.89	1.80
15.0	35.77	25.23	4.00	25.60	11.92
16.0	22.79	18.02	4.50	22.58	17.12
17.0	9.67	13.52	5.00	15.05	27.92
18.0	2.66	3.60	5.50	5.52	14.41
19.0	0.17	0.90	6.00	4.02	13.52
20.0			6.50	0.99	7.20
21.0	0.17		7.00	0.17	1.80
22.0	0.17		7.50	0.17	1.80
			8.00		0.90



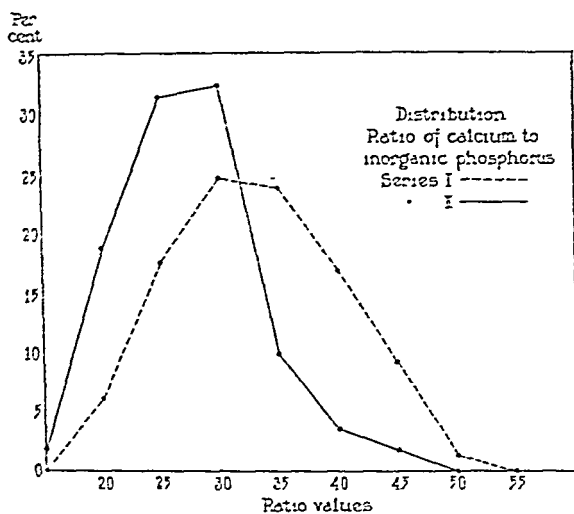
TEXT-FIG. 3.



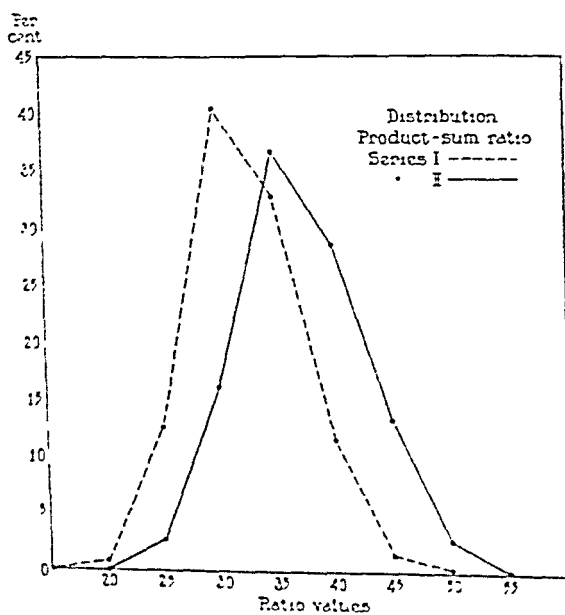
TEXT-FIG. 4.

TABLE IV.
Distribution of Values for Sum and Product.

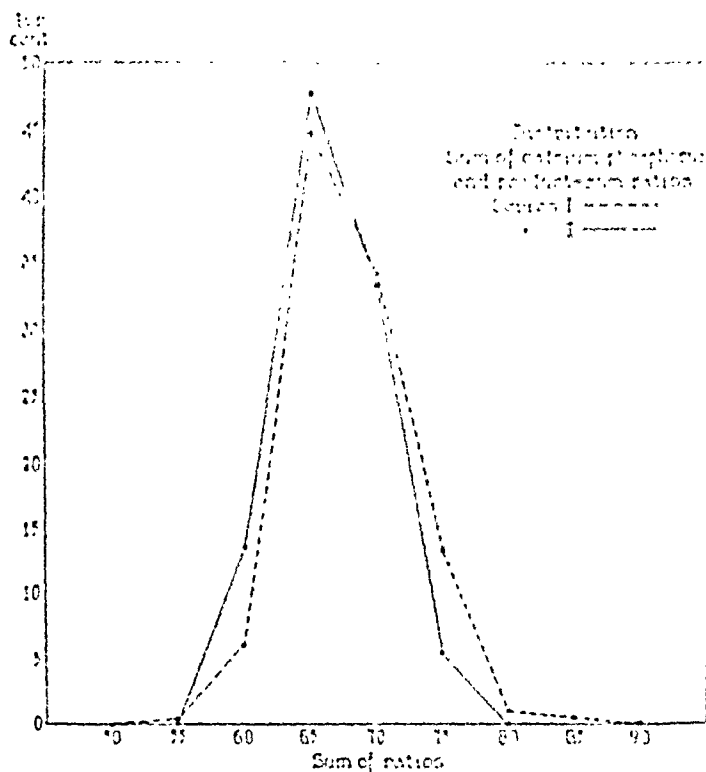
Sum values	Series I	Series II	Product values	Series I	Series II
	<i>per cent</i>	<i>per cent</i>		<i>per cent</i>	<i>per cent</i>
15.0	0.51		35.0	1.00	
16.5	4.86	4.50	45.0	8.53	2.70
18.0	24.79	16.22	55.0	24.30	7.20
19.5	45.39	34.23	65.0	33.67	22.52
21.0	19.44	27.03	75.0	21.28	25.22
22.5	3.35	13.52	85.0	8.04	20.72
24.0		3.60	95.0	2.51	10.81
25.5	0.17	0.90	105.0	0.66	5.40
27.0	0.17		115.0		4.50
			125.0		0.90



TEXT-FIG. 5.



TEXT-FIG. 6.



TEXT-FIG. 7.

TABLE V.

Distribution of Values for Ratios of Calcium to Inorganic Phosphorus and Product to Sum and for the Sum of the Two Ratios.

Calcium-phosphorus ratio values	Series I	Series II	Product-sum ratio values	Series I	Series II	Values for sum of two ratios	Series I	Series II
	per cent	per cent		per cent	per cent		per cent	per cent
1.50		1.80	1.50			5.50	0.34	
2.00	6.20	18.92	2.00	0.84		6.00	6.03	13.51
2.50	17.59	31.53	2.50	12.73	1.80	6.50	44.73	47.75
3.00	24.63	32.43	3.00	40.54	16.21	7.00	34.17	33.34
3.50	23.79	9.91	3.50	32.49	36.93	7.50	13.22	5.40
4.00	16.76	3.60	4.00	11.72	28.83	8.00	1.01	
4.50	9.21	1.80	4.50	1.51	13.52	8.50	0.51	
5.00	1.34		5.00	0.17	2.70			
5.50	0.17							
6.00	0.34							

DISCUSSION AND CONCLUSIONS.

From an analysis of the results obtained by repeated determinations of calcium and inorganic phosphorus in the blood of 4 groups of rabbits, it was found that, under the conditions employed, both the absolute and relative amounts of these substances were subject to considerable variation. The question to be determined by the present investigation is whether a change in the experimental conditions which eliminates the factors of increasing age and prolonged cage life in the laboratory tends to produce results of the same or of a different order.

The mean value for calcium (Table I) is well within the limits established for animals living under laboratory conditions, and the value for the sum of the calcium and inorganic phosphorus is the same as the highest mean value for the previous series, but in no other case does the mean for the present group of animals fall within these limits. The mean value for inorganic phosphorus is considerably higher; the product and the ratio of the product to the sum are also higher, while the ratio of calcium to phosphorus and the sum of the two ratios are lower.

In the case of calcium, the difference between the mean and the mean of the combined values of Series I is 0.2 mg. This difference is of the same order as that shown by the highest and lowest mean values of Series I as compared with the combined value, but is less than the extreme difference of 0.5 mg. One may conclude, therefore, that the mean value obtained for calcium, in the present instance, does not differ significantly from values obtained under the other experimental conditions (Tables I and II).

The difference between the mean value for inorganic phosphorus and the basic value for Series I is, however, greater than that between the highest and lowest mean values of the series, and is approximately twice as large as the difference between these values and the combined value for the series (Tables I and II). The value for inorganic phosphorus is definitely outside of the limits established for Series I, and the difference between the two sets of results is many times the probable error; so that, in this case, it is certain that the higher value for inorganic phosphorus is significant. In this connection, it is of interest to note that the highest value of Series I was given by the

youngest animals, and that the observation period on these animals, or the period of confinement in the laboratory, was the shortest.

A similar comparison of the figures given for the sum of calcium and inorganic phosphorus shows that the value obtained represents a deviation from our basic value which is comparable to that found for animals living under laboratory conditions. The mean value is in fact identical with that for the youngest group of animals so living, but the results differ in other respects as will be pointed out later.

The increase of the product value above the standard mean (Series I) is approximately equal to the extreme difference between group means of Series I and nearly twice the deviation shown by high and low values of the series from the standard mean. The shift in the product values assumes the same direction as that for the inorganic phosphorus content of the blood and has virtually the same significance.

The deviation of the calcium-phosphorus ratio from the combined value of Series I is greater than that shown by any of the groups in that series, and represents a definite downward shift. The ratio of the product to the sum changes nearly, but not quite as much in the opposite direction, giving a value for the sum of the two ratios which is again well below the limits for Series I.

The probability of the chance occurrence of differences between mean values of the magnitude of those obtained is indicated in Table II.

A comparison of the variability and of the distribution of the two sets of calcium and phosphorus determinations is just as instructive as a comparison of mean values, but no attempt will be made to follow this out in detail. By reference to Table I, it will be seen that, in most cases, the standard deviation for Series II exceeds the maximum value for Series I, while the coefficients of variation show a closer agreement and are in most cases of a comparable order.

The distribution frequencies are especially interesting in that they show differences in the composition of the two sets of results that are not brought out in any other way (Tables III to V and Text-figs. 1 to 7). For example, while the mean values for calcium show a comparatively close agreement, the composition of these means is quite different (Text-fig. 1). The distribution of individual determinations

covers essentially the same range, and both curves are fairly symmetrical. But, in Series I, a higher proportion of values is grouped about the mean, while in Series II the percentage of values in both the upper and lower ranges is greater, giving a larger standard deviation and a larger coefficient of variation.

In all other cases, irrespective of the agreement between means or of differences in variability, the range covered by the observations on Series II differs from that of Series I. The form of the curves is much alike, indicating that they are produced by variants of the same class, but the axis of distribution moves upward or downward on the scale of values, showing that there are quantitative differences in the composition of the two sets of results. The curves for the sum of the ratios of calcium to inorganic phosphorus and of the product to the sum show the closest agreement, but the agreement is not as close as that shown by the 4 groups of Series I. It appears, therefore, that even in this case there is a significant difference, and this supposition is borne out by the fact that the difference between mean values is 6 times its probable error.

Comparison of the two sets of results leads to the conclusion that the calcium and inorganic phosphorus in the blood of rabbits, as they are brought into the laboratory, may differ to a considerable extent from that of rabbits living under laboratory conditions. Grant and Gates (2) found that caging for periods as long as 7 weeks increased the calcium content of the blood, but in the present instance inorganic phosphorus and the relations between calcium and phosphorus were affected more than the absolute values for calcium. The evidence presented in this paper is not sufficient to warrant any conclusion concerning the extent to which age as distinguished from living conditions may have affected the results. The influence of these and other factors is shown to better advantage by comparing the results of consecutive observations on the two series of animals. This aspect of the investigation will be presented in a subsequent paper.

SUMMARY.

Determinations of calcium and inorganic phosphorus were made on the blood of a large series of rabbits as they were received from the

dealer for the purpose of determining whether results obtained under these conditions differed from those obtained by repeated observations on animals living in the laboratory.

It was found that the results differed in the two cases, due largely to a decided difference in the inorganic phosphorus content of the blood; but the values for calcium were also affected.

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THE INTERCONVERTIBILITY OF "R" AND "S" FORMS OF PNEUMOCOCCUS.

By MARTIN H. DAWSON,* M.D.

(From the Hospital of The Rockefeller Institute for Medical Research.)

(Received for publication, January 3, 1928.)

The terms "R" ("rough") and "S" ("smooth"), to designate two "variants" of the same bacterial species, were first employed by Arkwright (1). His observations were made on members of the colon-typhoid-dysentery group but were soon extended to many other organisms. Griffith (2) was the first to recognize two corresponding "variants" of *Pneumococcus*, and his findings were confirmed and extended by Reimann (3) and Amoss (4). The distinguishing features of the two forms of *Pneumococcus* may be summarized as follows: "S" forms are virulent; they produce the specific soluble substance, upon which type specificity depends (5); and they form colonies which have a smooth surface when examined by reflected light. "R" forms are avirulent; they do not produce the specific soluble substance; and they form colonies which have a rough surface when similarly examined.

Since the recognition of these two forms, much interest has centered in the question of their interchangeability. It is obvious that this subject, as Hadley (6) points out, has considerable significance, not only in the problem of epidemiology, but also in the interpretation of bacterial mutation.

It is proposed to review here the question of the interconvertibility of the two forms only in so far as it applies to *Pneumococcus*.

As early as 1891, Roger (7) reported that *Pneumococcus*, when grown in the sera of inoculated animals, became attenuated in virulence. Issaefi (8), in 1893, however, showed that this apparent loss of virulence was due to the protective action of the serum present, since bacteria, freed from the serum in which they had been grown, showed no alteration in virulence. Neufeld (9), in 1902, stated

* Fellow in Medicine of the National Research Council.

that a virulent strain of *Pneumococcus*, in the course of cultivation, may lose its virulence and its specific agglutinability. Friel (10), in 1915, found that pneumococci, when grown in homologous immune serum, became agglutinable and phagocytatable in normal rabbit serum, and less virulent for mice than the untreated strains. Stryker (11), independently, about the same time, reported that the growth of virulent pneumococci in homologous immune serum produced (a) variations in agglutinability, (b) decrease in virulence, (c) inhibition of capsule formation, (d) increased phagocytability in normal serum, and (e) a change in antigenic properties. She found that the modified type was permanent while the culture was kept on plain media; but that reversion to the original type occurred following animal passage. These investigations preceded the recognition of "R" and "S" forms but the indications are that the avirulent organisms were of the "R" variety. Griffith (2), in the paper in which he first pointed out the morphological distinction between colonies of virulent and attenuated pneumococci, stated that, "an 'R' strain may revert in all respects to the 'S' type or may remain unchanged after many generations in subculture in plain blood broth." He was of the opinion that the constancy of the "R" variant was, in some degree at least, dependent upon the strength of the immune serum employed in its production. None of these investigators made use of single cell cultures, and the contrary results subsequently obtained by Amoss (4) and Reimann (12), were, in part, attributed by them to the fact that they employed pure line strains derived from single cell cultures. Amoss reported that the avirulent strains showed no tendency to revert to the parent type, and did not become virulent on repeated passage through mice. Reimann was unable to restore virulence or type specificity to a single cell "R" strain even after 105 mouse passages. Both Reimann and Amoss employed "R" cultures derived from Type I pneumococcus. Felton and Dougherty (13), by means of an automatic transferring device in a milk-containing medium, were able to restore virulence to a single cell culture of Type I pneumococcus which had become completely avirulent. Although no mention was made of "R" and "S" forms in their communication, it may be assumed that the avirulent cultures were of the "R" variety. They reported, however, that some cultures showed a maximal increase in virulence while others remained avirulent. Levinthal (14) also reported that he was able to effect reversion from "R" to "S" in single cell Type I cultures by growth in serum broth at 25°C., with subsequent passage of the cultures through mice.

The present study concerns itself, generally, with the question of the interconvertibility of "R" and "S" forms of *Pneumococcus*, and, more particularly, with the question of reversion from "R" to "S." In a previous communication (15), it was pointed out that virulence and type specificity could be restored to "R" forms by growth in anti-"R" sera. Interesting in this connection is the observation of Soule

(16). Investigating microbic dissociation in *B. subtilis*, he stated that, "by the incorporation of 'S' or 'R' immune sera in fluid media, 'R' forms may be obtained from 'S' forms, and 'S' forms from 'R' forms respectively."

Methods.

Both mass and single cell cultures of Types I, II, and III pneumococcus were employed in all experiments. The single cell strains were isolated according to the method of Avery and Leland (17). In this method the position of single organisms is first accurately determined on a thin film of agar spread on a specially designed cover-slip. The single organisms are then allowed to develop into colonies which are subcultured as desired.

The structure and surface appearance of colonies were studied on blood agar plates by means of a Zeiss binocular microscope specially adapted for the study of colony morphology. It is necessary to point out that colony appearance is only a relative guide to the nature of the organisms constituting a colony, and can never be absolutely relied upon to distinguish "R" and "S" forms. This is particularly true in the case of Type II pneumococcus. In this study, colony morphology alone was never considered as a final criterion, but was always confirmed by specific agglutination and virulence tests. The appearance of colonies also shows great variation with age, so that, except in certain instances, plate cultures 18-24 hours old were always selected for examination.

The immune sera employed were either the type-specific, diagnostic sera prepared from horses by the New York State Department of Health,¹ or sera obtained from animals immunized according to the method of Cole and Moore (18).

Unless otherwise indicated, subcultures were made twice in 24 hours, and incubated at 37°C.

EXPERIMENTAL.

I. Reversion from "R" to "S" in Mass Cultures.

A. By Animal Passage.

Mass cultures of "R" forms derived, respectively, from pneumococci of Types I, II, and III, were employed. In each instance these were stock laboratory "R" strains which had been maintained in artificial cultivation at least 2 years, and which showed no tendency to revert to the "S" form by growth in any of the usual media. These

¹ We are indebted to Dr. Augustus B. Wadsworth of the New York State Department of Health for the antipneumococcus horse sera employed in this investigation.

cultures were avirulent, 0.5 cc. of a young broth culture uniformly failing to kill white mice; they did not form capsules or produce the specific soluble substances; and formed only typical rough colonies on blood agar plates.

(a) "*R*" Forms Derived from Type I (Strain I/192/R).—It was not found possible to effect reversion of this strain by animal passage. This confirmed the results of Reimann, who had previously subjected the same strain to 105 consecutive mouse passages.

(b) "*R*" Forms Derived from Type II (Strain D/39/R).—3 cc. of a 6 hour growth in plain broth were injected into the peritoneum of a mouse and the animal killed in 4 hours. 1 cc. of the peritoneal washings was injected into a second mouse, and the process repeated after 6 hours, the third animal succumbing on the following day. Three further passages were made and a blood broth culture from the sixth mouse proved highly virulent, 0.000001 cc. causing death of an injected animal. The culture now showed only typical "S" colonies, gave a typical Type II agglutination, and produced an abundance of the specific soluble substance. Reversion to the "S" forms had been effected in all respects.

(c) "*R*" Forms Derived from Type III (Strain M/3/R).—Much greater difficulty was experienced in causing this strain to revert and it was only after twenty-eight successive mouse passages that it was accomplished. However, reversion did ultimately occur, and it was accompanied by the acquisition of all the characteristics of the "S" form, including maximal virulence.

B. By Growth in Anti-"R" Sera.

The same three "R" strains, derived from Types I, II, and III, respectively, were grown in plain broth, to which 10 per cent of anti-"R" serum obtained from a rabbit immunized to an "R" form was added. In the first experiments the serum employed was from an animal immunized to an "R" form derived from Type III (Strain M/3/R), but it was subsequently found that any anti-"R" serum, provided it was of sufficiently high titer, was equally effective. This phase of the problem will be discussed later.

(a) "*R*" Forms Derived from Type I (Strain I/192/R).—No change was effected in this culture even after 100 transfers in 10 per cent

anti-"R" serum. The surface of the colonies became somewhat less rough but there was no increase in virulence and no evidence of return to type specificity.

(b) "*R*" Forms Derived from Type II (Strain D/39/R).—This strain, which had remained unchanged after many transfers in blood broth, but which could be reverted to the "S" type by animal passage, showed an unexpected transformation when grown in anti-"R" serum. During four transfers there was no change in the character of the growth, the organisms settling to the bottom of the tube in agglutinated masses, as is usual with "R" forms when grown in anti-"R" sera. On the fifth transfer, however, the media became slightly turbid, and more definitely so on the sixth subculture. On the seventh transfer, the culture showed a uniform turbidity, gave a specific Type II reaction, and proved highly virulent for white mice, 0.000001 cc. causing death. Examination of the colonies on plates showed only typical "S" forms. Evidently complete reversion had been effected.

(c) "*R*" Forms Derived from Type III (Strain M/3/R).—"R" forms of Type III pneumococcus reverted to the "S" type, when grown in anti-"R" sera, in a manner similar to "R" forms of Type II. After a variable number of transfers, usually between eight and twelve, the growth became diffuse, smooth colonies appeared on plates, and type specificity, accompanied by maximal virulence, was restored. It is of interest to note that, once the process was initiated, it was only a matter of two or three further transfers before the entire culture had assumed the "S" form, and "R" colonies could not be found.

From these experiments the conclusion may be drawn that, in certain instances at least, mass cultures of avirulent, "R" forms of pneumococci possess the ability to revert to virulent, type-specific, "S" forms, and that the change can be effected by *in vitro* as well as by *in vivo* methods.

Two questions then presented themselves: first, is reversion due to the presence of certain individual, undetected "S" forms within the mass "R" cultures, or do all "R" cells individually possess the ability to revert; and secondly, can reversion be effected by growth in an artificial medium other than that containing anti-"R" serum?

II. Reversion from "R" to "S" in Single Cell Cultures.

Four single cell strains were obtained from each of the three "R" cultures originally derived from the three specific types of *Pneumococcus*, and their characteristics definitely determined. They were avirulent, 0.5 cc. of broth culture producing no effect on injection into white mice; they showed no evidence of type specificity, and formed only rough colonies on plates. Reversion was then attempted both by animal passage and by growth in anti-"R" sera.

A. By Animal Passage.

The results obtained when single cell cultures were employed so closely parallel those outlined in the case of mass cultures that it is not necessary to give the data in detail. In no instance was it possible by animal passage to effect reversion with the single cell "R" forms derived from Type I (Strain I/192/R). The single cell "R" cultures derived from Types II and III (Strains D/39/R and M/3/R) reverted to the "S" form after practically the same number of mouse passages as was necessary when mass cultures were used. Little or no individual differences were found to exist in the various single cell cultures derived from the same "R" strain.

B. By Growth in Anti-"R" Sera.

The same dilution of serum was used which has been effective in producing reversion in mass cultures. Again entirely similar results were obtained, and practically no individual difference was found to exist in the various single cell strains isolated from the same mass culture. The "R" forms of Type I (Strain I/192/R) uniformly failed to revert; all the "R" forms of Type II (Strain D/39/R) reverted in from five to ten transfers; and the "R" forms of Type III (Strain M/3/R) in from ten to twenty transfers. The "R" forms of Type I were again carried in subculture up to 100 transfers in media containing anti-"R" serum without success.

From the results with single cell cultures, it seems fair to conclude that the reversion of an "R" strain to the "S" type does not depend upon the presence of an admixture of both forms within the culture, but rather it seems not unlikely that each individual "R" organism either may, or may not, possess the ability to revert to the "S" type.

Results with "R" Strains Derived from Other Type I Cultures.

The "R" strain of Type I which was used in the preceding experiments was a stock strain which had been under artificial cultivation for a period of many years. In order to determine whether this inability to revert was a property of all "R" organisms derived from Type I, or only of this particular strain, other "R" cultures derived from Type I were employed. These "R" forms were obtained by growing two Type I "S" strains, recently isolated from cases of lobar pneumonia, in 25 per cent Type I serum. After eight transfers in this medium, four typical "R" colonies, two from each strain, were selected; and reversion was attempted both in mass cultures grown from the single colonies, and in single cell cultures derived from the same colonies. That these cultures were definitely of the "R" forms was repeatedly confirmed by colony appearance, lack of type-specific agglutination, and loss of virulence. In this instance, uniform results were not obtained; but it is of interest to record that the single cell cultures behaved in precisely the same manner as the whole colony cultures from which the single cells were derived. Of the four colony cultures, it was possible, by the method of animal passage, to restore type specificity and virulence to only one. Likewise it was possible to effect reversion by animal passage in single cell cultures derived from this colony and not in the case of the others.

Reversion of these "R" strains of Type I was then attempted in anti-"R" sera. Success was attained with the culture whose virulence could be restored by animal passage but not with the others. Forty transfers in anti-"R" serum broth were necessary and both single cell and mass cultures reverted. Here, again, reversion was accompanied by the acquisition of all the characteristics of the "S" type, including maximal virulence. Thus it appears, at least in so far as Type I is concerned, that there are varying degrees of constancy of the "R" variant. Whether this constancy depends upon the strength of the immune serum employed in the production of the "R" forms as Griffith (2) suggests, or upon the number of transfers in immune serum to which the "R" variant has been exposed, further work is necessary to determine. So far, however, it has always been found possible to effect reversion of "R" forms of Types II and III, single cell as well as mass cultures, both by animal passage and by growth in anti-"R" sera.

Conversion of "S" to "R".

It was thought that possibly some light could be thrown on the difficulty experienced in causing "R" forms of Type I to revert to the "S" type by studying the reverse process ("S" \rightarrow "R") in greater detail than had previously been done. It was hoped that this procedure would also be of assistance in the interpretation of certain puzzling forms of colonies which were occasionally encountered when "R" forms were grown in anti-"R" sera. "S" strains of Types I, II, and III, were grown through repeated transfers in broth containing the homologous antisera in dilutions varying from 1:1, 1:2, etc., to 1:256.

Type I "S" (Strains I/192/2, "P," and "G").—Each strain went through a more or less similar transformation of which the following account of Strain I/192/2 is typical. Unless otherwise indicated, the description of colonies applies to plate cultures 18–24 hours old.

The growth in 100 per cent Type I serum was poor and plates showed only typical "S" colonies for three transfers. In all serum dilutions, where growth was more abundant, the colonies also remained typically "S" for three transfers before a change was produced. After the fourth transfer, a change became apparent in all dilutions from 1:2 to 1:64—there was great variation in the size of the colonies, without any change in their surface appearance or outline. In higher dilutions only typically "S" colonies occurred. On the fifth transfer, in dilutions 1:2 to 1:64, a small, flat, indistinct, rough colony made its appearance in frequent numbers. As these plates aged (48 hours) a most bizarre picture was produced. Many of the large, smooth colonies developed nibbled areas at the periphery, and the small, flat, rough ones produced a great variety of forms—some developed smooth, nodular papillæ; some a mulberry-like appearance; while others appeared to be undergoing autolysis. After the sixth transfer, the plate presented a still more unusual appearance. Many of the large, smooth colonies showed pronounced nibbling and irregularity of outline, while all the colonies showed a complete lack of uniformity in both size and surface appearance. Occasional "rough" colonies were observed. On the seventh transfer, the majority of the colonies possessed a definitely "rough" surface, but they showed a peculiar "watery" appearance which distinguished them from typical "R" forms. After the eighth transfer, typically "rough" colonies could be recognized but even up

to the fourteenth transfer the small, irregular, rough colony still persisted. Repeated attempts were made to isolate these small, irregular colonies and to establish stable, intermediate forms. These efforts were not successful. Subcultures showed mixtures of the atypical intermediate forms and true "S" colonies.

Type II "S" (Strain D/39/36/2).—In the case of Type II it becomes much more difficult to describe the colony appearance, for the distinction between "R" and "S" forms was much less pronounced and had a purely relative value. In fact it was not unusual to find that a virulent Type II culture would give rise to colonies which appeared rough; while, on the other hand, colonies which were apparently smooth, especially when grown in blood or serum broth, were not necessarily type-specific. However, it is possible to say that the transformation from Type II "S" to the "R" form occurred much more readily than in Type I, one exposure to type-specific serum often proving sufficient. Moreover, the unusual transition picture described in Type I was not encountered, possibly because of the readiness with which the transition to the "R" form occurred.

Type III "S" (Strain A66/47/2).—With Type III it was always possible to distinguish "R" and "S" colonies. After one transfer in homologous anti-"S" serum, in all dilutions, only "S" colonies occurred. After two transfers only a few "S" colonies persisted in serum dilutions 1:2 and 1:4; in higher dilutions only "S" forms were apparent. After four transfers, dilutions 1:2 and 1:4 showed only "R" forms; in dilution 1:8 only two "S" colonies remained. After the sixth transfer, all the colonies were of the "R" type in dilutions 1:2, 1:4, and 1:8, while in higher dilutions, only "S" forms persisted. No intermediate forms were observed, all colonies being definitely either "R" or "S."

From these experiments it is possible to state that Type I "S" pneumococcus is by far the most difficult to convert to the "R" form, requiring ten to fifteen transfers in serum dilutions of 1:2 and 1:4. Even after this number of transfers "intermediate" colonies exist which readily revert to the "S" type. On the other hand, Types II "S" and III "S" are easily converted to the "R" form, and in each instance the change is much more abrupt and complete than in the case of Type I. This fact may offer a partial explanation of the difficulty experienced in the reverse process of transforming "R" forms derived from Type I to the "S" type.

The Rôle of Anti-"R" Serum in Reversion by in Vitro Methods.

Having established the fact that, in the majority of instances, it is possible to transform "R" cultures of *Pneumococcus* to the "S" type by growth in anti-"R" sera, attempts were made to effect the change in media which did not contain anti-"R" antibodies. Experiments were also carried out to determine the titer and concentration of serum best suited for the purpose.

Sera were obtained from a series of normal individuals and animals, and also from animals which had been immunized to either the "R" or "S" forms of *Pneumococcus*. Attention is drawn to the observation of Avery and Heidelberger (19) that type-specific, antipneumococcus

TABLE I.
Reversion "R" to "S"—Type II Pneumococcus. Growth in Normal and Anti-"R" Sera.

Culture	Serum broth dilutions 10 per cent	Anti-"R" titer of sera	No. transfers	Result
"R" forms of Type II (D/39/R). Single cell cul- ture	Normal chicken	Nil	100	No change
	" rabbit	1:5	100	" "
	" sheep	1:20	100	" "
	" horse	1:20	100	" "
	" guinea pig	1:320	30	Partial reversion
	" human	1:640	8	Complete "
	Immune rabbit	1:3200	5	" "
	" horse	1:3200	5	" "
	Plain broth		100	No change

sera contain not only dominant, type-specific (anti-"S") antibodies, but also antibodies reacting with the protein substance, which is common to all pneumococci. Reimann (12) subsequently showed that sera prepared with "R" forms are immunologically similar to sera prepared with the protein of *Pneumococcus*. In consequence it was possible to use not only anti-"R" sera prepared against any "R" strain regardless of its type derivation, but also anti-"S" sera of the three specific types, all of which possess, in common, anti-"R" antibodies. The anti-"R" titer of the sera was determined by the so called thread reaction, and single cell cultures were employed.

The results obtained with "R" forms of Type II pneumococcus are set forth in Table I. It is seen that complete reversion occurred only in those sera of which the anti-"R" titer was above 1:320. In normal guinea pig serum, the titer of which was 1:320, a partial reversion was observed. Under these conditions the organisms lost their ability to thread; the colonies became smoother; and the virulence of the culture was increased, but not to a maximal degree. It was not probable in this instance that the growth was made up of a mixture of "R" and "S" forms; for all the colonies were of similar appearance and sub-

TABLE II.

Reversion "R" to "S"—Type III Pneumococcus. Growth in Normal and Anti-"R" Sera.

Culture	Serum broth dilutions 10 per cent	Anti-"R" titer of sera	No. transfers	Result
"R" forms of Type III (M/3/R). Single cell culture	Normal chicken	Nil	50	No change
	" goat	"	50	" "
	" horse	1:10	50	" "
	" sheep	1:20	50	" "
	" rabbit	1:20	25	Complete reversion
	" guinea pig	1:80	50	Partial " ?
	Immune goat	1:200	50	No change
	Normal human	1:640	50	" "
	Immune sheep	1:800	50	" "
	" rabbit	1:3200	18	Complete reversion
	" horse	1:6400	12	" "
	Plain broth		50	No change

cultures made from them individually possessed the same characteristics. Even when subcultured for twenty further transfers in the same media complete reversion did not occur. Growth in serum broth with an anti-"R" titer of less than 1:320 induced little change. In some instances the organisms ceased to give a thread reaction, but there was slight, if any, increase in virulence. They did not give a type-specific agglutination and were still unquestionably "R" forms.

The corresponding results obtained with "R" forms of Type III are outlined in Table II. Reversion again occurred promptly in those sera the anti-"R" content of which was highest, and not at all in those

which did not contain anti-"R" antibodies. A partial exception occurred in the case of normal rabbit serum, the anti-"R" titer of which was only 1:20. Here complete reversion was effected after twenty-five transfers. This was the only instance encountered in which the transformation took place in sera of low anti-"R" titer. This observation suggests that, while reversion unquestionably occurs much more readily in sera of high anti-"R" content, it may take place occasionally even in sera of low anti-"R" titer.

The results obtained by growing "R" forms of Type III in normal guinea pig sera are of some interest. A great variety of colony appearance was produced which closely resembled that previously described

TABLE III.
Optimal Concentration of Anti-"R" Serum for Reversion.

Culture	Serum concentration	No. transfers to effect reversion
"R" forms of Type II (Strain D/39/R)	25 per cent	8
	10 " "	4
	5 " "	10
	1 " "	No change
"R" forms of Type III (Strain M/3/R)	25 " "	16
	10 " "	7
	5 " "	20
	1 " "	No change

in the degradation of Type I "S" cultures. After fifteen transfers in this medium, colonies quite irregular in size, outline, and surface appearance were observed. In addition to the more usual "rough" forms there were small, indistinct, flat, smooth colonies; small, rough ones from which projected smooth nodular papillæ; and medium sized, apparently smooth colonies. All of these types of colonies appeared on plate cultures after 18 hours incubation. After ten further transfers only smooth colonies occurred but they were not of the large, mucoid, typical, Type III variety and subcultures from them were avirulent and not type-specific. Continued transfers in normal guinea pig serum produced no change and when transferred back to plain broth only typical "R" forms resulted. Possibly this culture represented an unstable intermediate form.

Optimal Concentration of Anti-"R" Sera for Reversion.

Cultures of "R" forms of Types II and III pneumococcus were grown for successive transfers in broth containing varying dilutions of anti-"R" rabbit serum of known titer. The results are recorded in Table III. The anti-"R" titer of the serum used, as determined by the thread reaction, was 1:6400. In all experiments 10 per cent was found to be the optimal concentration. Reversion also occurred in serum dilutions of 25 per cent and 5 per cent but a greater number of transfers was necessary.

Experiments were also done with sera which had been inactivated by heating at 56° for $\frac{1}{2}$ hour, and it was found that this procedure did not reduce the anti-"R" titer nor destroy its ability to cause reversion. No work was done under anaerobic conditions nor were the sera of animals immunized to the nucleoprotein of *Pneumococcus* tested.

DISCUSSION.

The present work establishes the fact, that, in the majority of instances, it is possible to cause both mass and single cell "R" cultures of *Pneumococcus* to revert to the "S" type. It was found that there are differences in the stability of the "R" forms in the three types, "R" forms of Type I being the most permanent. This corresponds to the greater resistance to degradation exhibited by Type I "S." Absolutely irreversible "R" organisms were encountered only in Type I. No attempt was made to determine whether it is possible to produce such irreversible forms in Types II and III.

It is possible to cause reversion from "R" to "S" pneumococci by an *in vitro* as well as an *in vivo* method. Reversion from "R" to "S," whether effected *in vitro* or *in vivo*, is always accompanied by the acquisition of maximal virulence. The *in vitro* method consists in growing "R" organisms in anti-"R" sera, the optimal concentration of which is 10 per cent. Growth in sera which do not contain anti-"R" antibodies, has, with one partial exception, not been found to be effective in producing the transformation. The partial exception was that of a normal rabbit serum, the anti-"R" titer of which, as determined by the thread reaction, was 1:20. "R" cultures have invariably reverted to the specific type of *Pneumococcus* from which the

STUDIES UPON THE PHYSIOLOGICAL ACTION OF HEMATOPORPHYRIN.

By HANS SMETANA.

(From the Department of Pathology, Peking Union Medical College, Peking, China.)

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INTRODUCTION.

The behavior of animals having non-pigmented skin when injected with a suitable dose of hematoporphyrin, is well known. If such an animal is kept in the dark, no striking difference in behavior occurs from that observed in a normal, uninjected animal kept under the same conditions. On the other hand, if an animal having received such a dose of hematoporphyrin is exposed to sunlight or to strong, well cooled artificial light it soon becomes restless, violently scratches the nose, ears and other parts of the body, frequently jumps high into the air and shows livid discoloration of the skin; the respiration is rapid and the pulse rate increased. Following a short period of such activity the animal grows very weak and listless, gradually falls into coma, develops slow, very deep respiration, a slow pulse, and soon dies. This physiological state has been called hematoporphyrin shock.

The anatomical changes taking place in an animal subjected to this experiment are quite inadequate to explain the cause of death, or to account for the nature of the rôle which either light or hematoporphyrin plays in the process.

Soon after exposure to light is begun, edema of the skin appears, in white mice first becoming manifest in areas unprotected by hair, such as the eyelids, ears and tail. Rigor mortis occurs rapidly. The blood is very dark and clots slowly after death. Hemorrhages may be found in the internal organs, and in some cases blood is present in the lumen of the intestine. The mesenteric vessels, which after death are always found to be distended with blood, occasionally show marked infiltration of their walls with leucocytes, but this finding is frequently absent in even the most severe cases. Slight infiltration of leucocytes in the edematous skin may occur.

Animals injected with such a fatal, sensitizing dose of hematoporphyrin and then exposed to diffused daylight do not develop the acute symptoms of acute hematoporphyrin shock just described. They show signs of irritation, and after repeated injections with hematoporphyrin and exposures to light, their ears become necrotic and drop off. Following a period of anorexia, such animals die, but show no significant or consistent anatomical changes. Alterations in the central nervous system have been described by Fippinger and Arnstein (1).

Shock is never produced by exposure of only a small portion of the body to sunlight after injection of hematoporphyrin. The skin of the exposed area, however, becomes edematous and red, and after a few days shows necrosis. A deep ulcer develops at this point which heals slowly. Microscopically one finds thrombosis of the small vessels in the margin of the ulcer, with necrosis and cellular infiltration of its surface.

After intravenous, intraperitoneal or subcutaneous injection, hematoporphyrin may soon be found in the stomach and intestine, bile and urine. The blood serum at this time contains only a faint trace of hematoporphyrin.

We thus see that hematoporphyrin in suitable doses, *per se*, produces no marked effect when injected into animals. When, however, such animals are exposed to sunlight soon after injection of the drug, very characteristic and striking symptoms appear which rapidly progress to a state of coma soon ending in death.

Anatomical studies performed upon such animals reveal no adequate explanation of the mechanism by which the combined action of light and hematoporphyrin produces death. Comparatively little is known of the physiological state of the body produced by the combined action of hematoporphyrin and light. As the result of a series of experiments performed *in vitro*, Gaffron (2) has concluded that hematoporphyrin acts as a catalyzer which, under the influence of light, causes acceleration of oxidation and accordingly rapid consumption of oxygen.

The following experiments have been performed in the attempt to elucidate further the physiological characteristics of the condition which has been described as hematoporphyrin shock, as well as to test the validity of the theory concerning the nature of the action of hematoporphyrin proposed by Gaffron (2).

All of the hematoporphyrin used in these experiments was prepared by the well known method of Nencki (3).

Effect of Feeding Hematoporphyrin.

An attempt to sensitize white mice to light by feeding hematoporphyrin was ineffective.

Four mice were fed upon bread only, soaked in a weak solution of hematoporphyrin and dried. Two of these animals were kept constantly exposed to diffused daylight in the laboratory for 2 months, while the other two were kept in the dark. Upon numerous occasions during this time all four mice were exposed to direct bright sunlight for several hours. No changes in the skin, general appearance or behavior were noted in any of the four animals. Hematoporphyrin was constantly present in the stools but was never found in the urine.

The Relationship of the Cutaneous Pigment to the Effect of Hematoporphyrin and Light.

Since it is well known that exposure to light after injection of hematoporphyrin affects only white animals or those whose skin is slightly pigmented, it was of interest to determine whether this protection is of purely physical nature or in some other way associated with the pigment.

Albino and slightly pigmented rats and mice were stained a dark blue-black color with Verhoeff's hematoxylin and injected, along with unstained controls, with doses of hematoporphyrin adequate to cause death of the controls upon exposure to sunlight. A number of both groups of animals were exposed to sunlight and the others kept either in the dark or in the subdued light of the laboratory. The unstained controls promptly developed hematoporphyrin shock when exposed to sunlight, while the darkly stained animals, which had just received the same amounts of hematoporphyrin as had the controls, were completely protected from the sun's rays. Repeated exposure of these animals to sunlight produced no untoward effects.

It was clear from this experiment that the same degree of protection offered by cutaneous pigment can be produced by staining the skin of non-pigmented animals. Accordingly, one seems justified in assuming that the natural pigment of the skin plays only a physical rôle in the protection of animals injected with hematoporphyrin from light.

None of the stained or control animals kept in the dark or in subdued light showed any changes in their general behavior. Frozen sections of the skin of these two groups of animals, as well as those exposed to sunlight and normal animals, were made and treated with dioxyphenylalanine (Dopa reaction) to see if any alteration in the cutaneous pigment had been produced by any of these procedures (4). No changes of this nature could be demonstrated.

Sensitizing Effect of the Blood of Animals in Hematoporphyrin Shock.

It has so far been impossible to demonstrate any sensitizing substance in the blood of animals during the period of hematoporphyrin shock.

White mice were injected intraperitoneally with 1.5 cc., or intravenously with $\frac{1}{2}$ cc. each, of the heart's blood of guinea pigs drawn at the height of hematoporphyrin shock and kept in diffused daylight. No difference could be seen between

the behavior and general appearance of these animals and mice similarly injected with an equal amount of normal guinea pig blood and kept under the same experimental conditions. All of these mice were very ill for a few days following the injection, but recovered.

Since injection of blood from another species was obviously undesirable, the sensitizing effect of the blood of animals in hematoporphyrin shock was tested in another way.

A parabiosis of two young white rats of the same litter and sex was performed. 6 weeks after the operation, when both rats were in good condition, one of these animals was injected intravenously with trypan blue. The skin of the non-injected rat, as well as that of the injected one, soon became colored by the dye, thus proving the existence of adequate vascular connections between the circulations of the two animals. The first rat was then injected subcutaneously with 4 cc. of a 1 per cent alkaline solution of hematoporphyrin and exposed to bright sunlight, while the second rat was protected from the rays of the sun by a wooden box which was cut out on one side so as not to obstruct the blood vessels connecting the circulations of the two animals. The injected and exposed rat soon showed the characteristic symptoms of sensitization to light, passed into hematoporphyrin shock and died. The other animal, protected from the light, showed no changes in appearance or behavior. Just before the death of the first rat, the two animals were separated and the wound in the side of the unexposed rat closed. This animal lived and appeared to be entirely normal during the several months it was kept in the laboratory. No pathological changes were found at autopsy.

Exposure of the Peritoneum to Light after Injection of Hematoporphyrin.

Two cats of about equal size were used for this experiment. One animal was given subcutaneously 15 cc. of 1 per cent alkaline hematoporphyrin and shortly afterwards, under ether anesthesia, the peritoneal cavities of both animals were opened widely, and exposed to bright sunlight. The peritoneum was kept moist with normal saline, and the rest of the body well protected from the light by towels. After 3 hours exposure of the peritoneal cavity to sunlight, the cat which had been injected with hematoporphyrin died. At this time the peritoneal cavity of the control animal, which was still in good condition, was closed and the anesthesia stopped. This latter animal quickly recovered.

This single experiment suggests that exposure of any other large surface of the body to sunlight after the injection of hematoporphyrin has the same effect as the exposure of non-pigmented skin. It was observed that the serosa of the intestines of the injected cat became very pale during the exposure to sunlight, while that of the normal animal showed marked hyperemia.

Effect upon the Blood Pressure of the Exposure to Strong Light of (a) Circulating Blood of Normal Animals, (b) Circulating Blood after Injection of Hematoporphyrin.

(a)

Reed (5), experimenting with normal dogs, reports a marked fall of blood pressure, sometimes reaching shock level, upon exposure to strong arclight of the blood alone. He interposed a glass tube, 3 mm. in diameter, between the divided

TABLE I.

(a) Exposure to Arclight and Sunlight of Circulating Blood of Normal Animals.

Type of experiment	Animal	Hours of exposure	Exposure	Mean blood pressure		Fall in blood pressure	Remarks
				Beginning of experiment	End of experiment		
			mm. Hg	mm. Hg	mm. Hg	mm. Hg	
Cannula interposed in femoral artery or connecting femoral artery and vein exposed to arclight	Dog 1	4.40 p.m.-7.10 p.m.	150	115	80	35	
	Dog 2	4.16 p.m.-5.30 p.m.	74	135	100	35	
	Dog 5	3.20 p.m.-4.40 p.m.	80	140	110	30	
	Cat 101	3.15 p.m.-5.00 p.m.	105	140	60	80	
Cannula connecting femoral artery and vein exposed to sunlight	Dog 8	11.16 a.m.-12.45 p.m.	89	130	130	0	Jan.
	Cat 21	11.16 a.m.-1.15 p.m.	119	110	60	50	May
	Cat 24	10.52 a.m.-12.51 p.m.	119	110	60	50	May (spiral cannula)
	Cat 27	10.11 a.m.-11.40 a.m.	89	150	100	50	May (spiral cannula)

ends of the carotid artery, and exposed the blood flowing through this tube to a beam of light supplied by a powerful carbon arc. In some cases the tube was cleaned repeatedly during the experiment, while in others coagulation of the blood was prevented by injection of heparin. Reed remarks that the result was somewhat modified at times by the use of heparin.

In repeating Reed's experiments in slightly modified form upon cats and dogs such a great fall in blood pressure as he reports has not been observed.

In our experiments a quartz glass tube was either interposed between the cut ends of the divided femoral artery of the anesthetized animal, or it was used to connect the femoral artery and vein. Coagulation of the blood was prevented by injection of heparin. The blood of cats and dogs while flowing through such a tube was exposed, in some cases, to a cooled beam of light composed of the rays

TABLE II.

(b) *Exposure to Arc-light and Sunlight of Circulating Blood after Injection of Hematoporphyrin.*

Type of experiment	Animal	Hours of exposure	Exposure min.	Mean blood pressure		Fall in blood pressure mm. Hg	Amount of hematoporphyrin injected
				Beginning of experiment mm. Hg	End of experiment mm. Hg		
Cannula femoral artery and vein. Intraven. inj. hematoporphyrin. Cannula exposed to arc-light	Cat 20 (1,800 gm.)	4.00 p.m.- 5.50 p.m.	110	135	30	105	Intraperiton. inj. 20 cc. 0.5% hematoporphyrin
	Cat 105 (2,100 gm.)	11.20 a.m.- 1.30 p.m.	130	100	65	35	Intraven. inj. 15 cc. 0.8% hematoporphyrin
	Dog 2 (5,200 gm.)	5.30 p.m.- 7.10 p.m.	100	100	90	10	Intraven. inj. 15 cc. 4% eosin
Cannula femoral artery and vein. Intraven. inj. hematoporphyrin. Cannula exposed to sunlight	Cat 22 (1,900 gm.)	11.30 a.m.- 1.55 p.m.	145	120	80	40	May, 20 cc. 0.5% hematoporphyrin
	Cat 23 (1,850 gm.)	11.30 a.m.- 2.00 p.m.	150	120	65	55	May, 20 cc. 0.5% hematoporphyrin (spiral cannula)

from a 1000 watt carbon arc, and in other instances to sunlight. The mean blood pressure was recorded directly from the carotid artery.

As seen in Table I, there occurred a considerable fall in mean blood pressure in all cases except Dog 8, in which no fall in pressure followed

89 minutes exposure of the circulating blood to direct sunlight. In no instance, however, did the mean blood pressure fall below 60 mm. Hg. In the experiments performed upon Cats 24 and 27, a spiral cannula, 3 mm. in diameter, was used as an exposure chamber for the blood instead of the simple glass tube employed in the other experiments. In this way, a much larger quantity of blood was constantly exposed.

From this group of experiments, therefore, it does not seem that exposure of the circulating blood alone to strong light produces any strikingly greater fall of arterial pressure than might be expected to take place following approximately 2 hours anesthesia carried out in the ordinary light of the laboratory.

(b)

In this group of animals, the preceding experiment was repeated immediately after the injection of a sufficient dose of hematoporphyrin to sensitize the animals to sunlight should the entire body be exposed. In all cases the bodies of the animals, however, were protected from the light, and only the blood circulating through the glass tube allowed to come in contact with the rays of light.

The results of this experiment, as summarized in Table II, show that in only one instance, Cat 20, did a greater fall of arterial pressure occur than took place in the normal animals receiving no injection of hematoporphyrin. In one case eosin, which has sensitizing properties similar to those of hematoporphyrin, was injected. None of these animals died during the experiment, but were killed when exposure of the blood to light was discontinued.

Changes Produced in Vitro by Light upon Blood Containing Hematoporphyrin.

Though no differences could be elicited between normal animals and those injected with a sensitizing dose of hematoporphyrin by exposing the blood flowing through a glass tube to strong light, it was not felt that this experiment adequately ruled out the possibility that the striking changes occurring in sensitized animals upon exposure of the body surface to sunlight, might be associated with changes taking place within the blood itself. Accordingly a series of experi-

ments was performed to test the direct effect of hematoporphyrin and sunlight upon blood *in vitro*.

Blood was obtained from both cats and dogs either by venipuncture or directly from the heart and mixed with a small amount of heparin or potassium oxalate to prevent clotting. Each sample was divided into three equal parts. Hematoporphyrin (0.002 gm. to 5 cc. of blood) dissolved in normal saline and a few drops of $N/10$ sodium hydroxide were added to two of the tubes, while to the third tube only similar amounts of saline and $N/10$ sodium hydroxide were added. Tubes 1 and 3 were exposed either to sunlight or to the well cooled rays of the 1000 watt carbon arc. Tube 2 was always kept in the dark. The blood in Tube 1, containing hematoporphyrin, invariably changed to a very dark, brownish red color upon

TABLE III.

Changes in the Oxygen Capacity and Content of Blood Produced in Vitro by Addition of Hematoporphyrin and Exposure to Sunlight.

Tube 1		Tube 2		Tube 3	
Blood + hematoporphyrin Exposed to sunlight		Blood + hematoporphyrin Kept in dark		Blood + hematoporphyrin Exposed to sunlight	
O ₂ capacity	O ₂ content	O ₂ capacity	O ₂ content	O ₂ capacity	O ₂ content
rel. %	rel. %	rel. %	rel. %	rel. %	rel. %
18.1	6.5	18.0	15.7	18.1	16.5
19.0	6.2	17.0	12.0	17.1	16.8
17.0	2.0	17.5	12.0	17.5	12.0
	3.4		13.0		13.4

exposure to light, the change becoming more marked as the exposure was prolonged. The blood in Tube 2, containing an equal amount of hematoporphyrin, but kept in the dark, showed no change of color. Tube 3, which contained no hematoporphyrin, did not show any change of color even after prolonged exposure to bright sunlight.

It is clear, therefore, that blood to which hematoporphyrin has been added changes to a dark, brownish red color upon exposure to sunlight, and that no such change occurs if such blood is kept in the dark. In the attempt to learn the reason for the striking change of color produced by sunlight in blood containing hematoporphyrin, the oxygen and carbon dioxide contents, as well the oxygen capacity of the three varieties of blood mentioned above were determined by the methods of Van Slyke (Tables III and IV).

From these two tables it will be seen that a marked diminution of oxygen content and increase of carbon dioxide content regularly occurred in the blood of Tube 1, which contained hematoporphyrin and had assumed a dark brownish red color upon exposure to sunlight. The oxygen and carbon dioxide contents of Tube 2, containing blood and hematoporphyrin, which was kept in the dark for the same period that Tube 1 was exposed to sunlight, remained essentially the same as Tube 3, which contained only normal blood without hematoporphyrin, but which was exposed to sunlight along with Tube 1. No changes in color were ever noted in Tubes 2 and 3. The oxygen capacity of all three samples of blood was always approximately the same. Exposure

TABLE IV.

Changes in the Carbon Dioxide Content of Blood Produced in Vitro by Addition of Hematoporphyrin and Exposure to Sunlight.

Tube 1	Tube 2	Tube 3
Blood + hematoporphyrin Exposed to sunlight	Blood + hematoporphyrin Kept in dark	Blood + hematoporphyrin Exposed to sunlight
Carbon dioxide content	Carbon dioxide content	Carbon dioxide content
rel. %	rel. %	rel. %
32.1	20.8	19.0
28.8	20.8	17.8
25.0	16.8	18.8

of the surface of the blood in the test-tube to the air did not appear to influence the character of the changes regularly observed to occur in Tube 1. This was demonstrated by covering the surface of the blood in all three tubes with a layer of paraffin oil in about half of the experiments.

Causes for Change in Color of Blood Containing Hematoporphyrin When Exposed to Sunlight.

It seems clear that the diminution of oxygen and increase of carbon dioxide content, which has been shown to take place in blood containing hematoporphyrin when exposed to light *in vitro*, are the principal factors which account for the alteration of color of such blood taking place under these conditions. Such qualitative changes, however,

could hardly be held responsible for the distinct brownish tinge which invariably accompanies the darkening in color of such blood.

It was suspected that the brownish color might be due to the presence of methemoglobin, but this was quickly shown to be untrue by repeated spectroscopic examinations of different samples of blood in which the characteristic color changes had occurred. In no instance could a trace of methemoglobin be detected.

TABLE V.
Resistance of New Blood Cells.

Per cent of NaCl in the test-tubes, . . .	0.28	0.30	0.32	0.34	0.36	0.38	0.40	0.42	0.44	0.46	0.48	0.50
Test-Tubes A containing blood and hemato- porphyrin exposed to light	+++	+++	+++	+++	+++	+++	+++	++	++	+	+	±
	+++	+++	+++	+++	++	++	++	+	±	±	±	±
	+++	+++	+++	++	++	++	++	+	±	±	±	±
Test-Tubes B containing blood and hema- tophyrin kept in dark	+++	+++	+++	+++	++	++	++	+	+	±	±	±
	+++	+++	+++	++	++	+	+	+	±	±	±	±
	+++	+++	++	++	++	+	+	+	±	±	±	±
Test-Tubes C containing blood without hema- tophyrin ex- posed to light	+++	+++	+++	++	++	++	+	+	±	±	±	±
	+++	+++	+++	++	++	++	+	+	±	±	±	±
	+++	+++	++	++	+	+	+	+	±	±	±	±

+++ complete hemolysis, ++ marked hemolysis, + hemolysis, ± trace of hemolysis.

After centrifugalization, the plasma of Tubes 1, 2 and 3 showed marked differences in color. That of Tube 1 was always very dark red, of Tube 2 a much lighter red, while the plasma of Tube 3 showed only a reddish tinge. Upon pipetting off the plasma and washing the red corpuscles with normal saline solution, the three tubes were again centrifugalized. The saline of Tube 1 was dark red, that of Tube 2 reddish tinged, while that of Tube 3 was colorless. Upon repeating this process upon the same three samples of red corpuscles,

the saline of Tube 1 was still red though of somewhat lighter shade than before, while the saline of both Tubes 2 and 3 remained colorless.

These findings suggested that an increase in fragility of the red blood corpuscles occurred in blood containing hematoporphyrin when exposed to sunlight *in vitro*. Fragility tests were accordingly performed upon the blood of three animals (guinea pigs) to test this hypothesis. Each sample of blood was divided into three equal parts and subjected to the same procedures as outlined above for Tubes 1, 2 and 3. The results of this experiment are summarized in Table V.

As will be seen from Table V, no appreciable difference in resistance could be demonstrated in the red corpuscles of the blood which contained hematoporphyrin and had been kept in the dark, and those of normal blood to which no hematoporphyrin had been added and which had been exposed to sunlight. On the other hand, a distinct increase in fragility of the red cells appeared to have taken place in the blood to which hematoporphyrin had been added before exposure to sunlight. It is at present not possible to state the mechanism by which this fragility of red blood cells is brought about by the combined action of hematoporphyrin and sunlight, but it seems clear that this phenomenon plays an important rôle in the staining of the plasma which occurs regularly under the above mentioned conditions.

The brownish tint observed after exposure to sunlight in blood containing hematoporphyrin is probably due largely to changes in the hematoporphyrin itself. When an alkaline solution of this substance is exposed to sunlight in a test-tube, its bright red color soon changes to a dark, brownish red. This change occurs either in an open test-tube, or when the solution is covered with a layer of paraffin oil to protect it from the air. Such a brownish red solution shows a slight spectroscopic difference from alkaline hematoporphyrin kept in the dark, an extra line appearing in the spectrum between *b* and *c*. Its physiological action, however, remains unaltered. The minimal lethal dose for mice, 0.015 gm., and the amount necessary to fatally sensitize mice to sunlight, 0.005 gm., are the same as for alkaline hematoporphyrin in which this change of color has not occurred.

The Blood Pressure in Hematoporphyrin Shock.

The blood pressure in the carotid artery of cats, under ether anesthesia, was recorded before, during and after the injection of sensitiz-

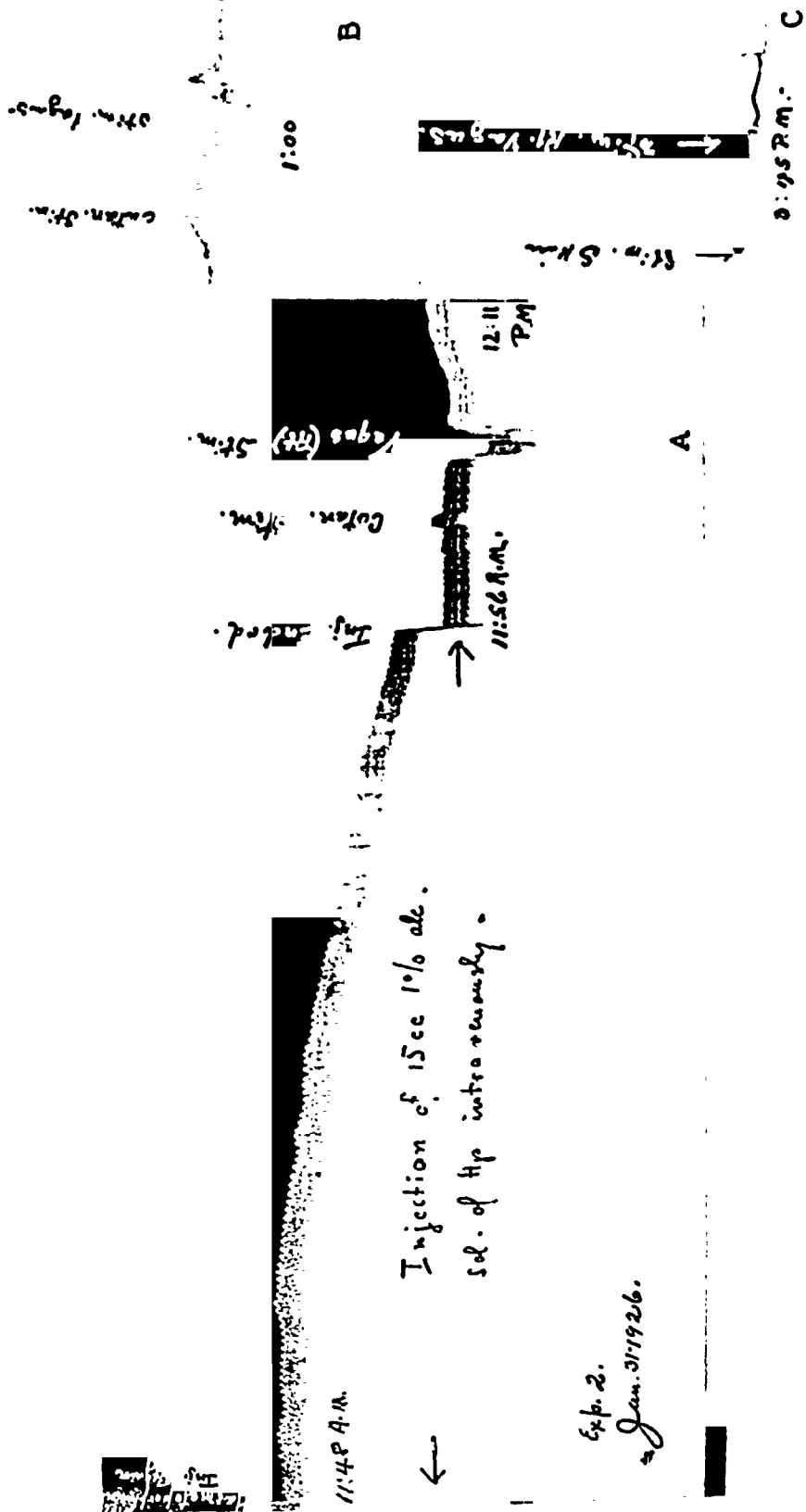


FIGURE 1. The blood pressure after injection of hematoporphyrin and exposure of the body to sunlight

ing doses of hematoporphyrin and exposure of the animals to direct sunlight. The crystalline hematoporphyrin was always dissolved in 1 to 2 per cent sodium bicarbonate, and 0.1 gm. of hematoporphyrin per kilo of body weight injected intravenously or subcutaneously.

TABLE VI.

Effect of Exposure to Sunlight upon the Arterial Blood Pressure of Cats Sensitized with Hematoporphyrin.

Animal No.	Date	Length of time of exposure	Exposure min.	Mean blood pressure		Fall in blood pressure	Remarks
				Before exposure to light mm. Hg	Just before death mm. Hg		
Cat 1 (15,508 gm.)	Jan. 17	12.30 p.m.- 1.58 p.m.	88	150	40	110	Intraven. inj. 15 cc. 1% hematoporphyrin
Cat 2 (2,650 gm.)	Jan. 31	11.48 a.m.- 1.06 p.m.	78	120	50	70	Intraven. inj. 20 cc. 1% hematoporphyrin
Cat 3 (2,065 gm.)	June 16	10.10 a.m.-10.45 a.m.	35	120	40	80	Subcut. inj. 25 cc. 1% he- matoporphyrin
Cat 25 (1,800 gm.)	May 10	11.00 a.m.-12.15 p.m.	75	120	40	80	Subcut. inj. 25 cc. 0.8% hematoporphyrin

After exposure of such an animal to direct sunlight, the respiration soon becomes more rapid, and the blood pressure falls steadily. After 30 to 60 minutes the respiration has generally become very deep, slow, irregular and labored. At this point the animal is in sufficiently deep coma for the anesthetic to be removed, and after a widely variable length of time (a few minutes to an hour or more) dies. The mean arterial pressure falls rapidly for a time, until the animal is in coma,

and then continues to fall gradually, reaching 40 or 50 mm. just before death (Table VI). The rapidity of the appearance of coma and the fall of blood pressure seems to be directly proportional to the intensity of light and the amount of hematoporphyrin injected. Respiration and heart beat cease simultaneously. Typical blood pressure responses to vagal and cutaneous stimuli were obtained throughout the experiment (Chart 1).

TABLE VII.

Effect Produced upon the Carbon Dioxide-Combining Power, Oxygen Content and Oxygen Capacity of the Blood of Guinea Pigs by Exposure of the Animals to Sunlight after Injection of Hematoporphyrin.

Normal animal exposed to sunlight			Animal injected with hematoporphyrin and kept in dark			Animal injected with hematoporphyrin and exposed to sunlight		
Carbon dioxide-combining power	Oxygen content	Oxygen capacity	Carbon dioxide-combining power	Oxygen content	Oxygen capacity	Carbon dioxide-combining power	Oxygen content	Oxygen capacity
rel. %	rel. %	rel. %	rel. %	rel. %	rel. %	rel. %	rel. %	rel. %
47.5	14.0	19.5	48.5	12.9	18.5	16.6	6.5	19.2
40.9	12.6	18.7	37.5	11.5	18.9	19.5	10.5	19.6
44.7	11.8	18.8	38.5	13.7	19.1	18.3	6.9	18.8
54.1						20.2	9.0	
						19.8		

Chemical Changes in the Blood of Animals during Hematoporphyrin Shock.

The following experiments, which were performed upon guinea pigs, were repetitions *in vivo* of the experiments performed *in vitro* upon mixtures of blood and hematoporphyrin already described.

Three animals were used in each experiment. Two of them were injected with a fatal, sensitizing dose of hematoporphyrin. One of these was kept in the dark, while the other, along with the third guinea pig, which had received no hematoporphyrin, was exposed to direct sunlight. Neither the injected animal kept in the dark, nor the normal guinea pig which was exposed to sunlight, showed any alterations in behavior, but the animal which had received hematoporphyrin and was then exposed to sunlight developed the characteristic symptoms of shock as produced under these conditions, and soon died.

Just before the death of this last animal, blood was drawn in a syringe from the right ventricles of the hearts of all three guinea pigs, and immediately placed

under paraffin oil without coming in contact with the air, coagulation being prevented by potassium oxalate mixed with the blood in the syringe. The carbon dioxide-combining power, as well as the oxygen content and oxygen capacity of each of these samples of blood, was determined.

In Table VII, it will be seen that there was little or no difference in the carbon dioxide-combining power of the blood of normal guinea pigs exposed to sunlight and that of animals injected with a fatal sensitizing dose of hematoporphyrin and kept in the dark. Those animals which had received an equal dose of hematoporphyrin and then been exposed to sunlight showed a marked reduction in carbon dioxide-combining power of the blood. No significant difference was found in the

TABLE VIII.

Effect Produced upon the Non-Protein Nitrogen, Creatinine and Sugar of the Blood of Guinea Pigs by Exposure of the Animals to Sunlight after Injection of Hematoporphyrin.

Normal animal exposed to light			Animal injected with hematoporphyrin and kept in dark			Animal injected with hematoporphyrin and exposed to light		
Creatinine	Sugar	N.P.N.	Creatinine	Sugar	N.P.N.	Creatinine	Sugar	N.P.N.
mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
2.0	200	27	2.5	160	33	2.3	250	26
1.5	181	33	2.5	166	31	3.0	285	31
1.3	154	32	2.7	285	37.5	2.5	166	35
2.1	222	31.6				2.3	106	42.8
3.0	200	46.1				1.5		
		37.6						

oxygen content of the bloods of normal guinea pigs exposed to sunlight, and those injected with hematoporphyrin and kept in the dark. Guinea pigs injected with hematoporphyrin and exposed to sunlight showed a marked diminution of oxygen in their blood. No change could be demonstrated in the oxygen capacity of blood from these three types of animals.

The non-protein nitrogen, sugar and creatinine of the blood taken from these three groups of animals were determined, but no significant alterations of the amounts of these substances under any of the experimental conditions were found. The results of these studies are summarized in Table VIII.

The red corpuscles and the leucocytes were counted in cats before the injection of hematoporphyrin, and during all stages of shock experimentally produced by this substance. The results of these counts have corroborated the observations of other workers. The red blood cells, which averaged about 8,800,000 per c.mm. in normal cats, showed no significant change during the entire period, which began with the injection of hematoporphyrin and ended with death. The leucocytes, which averaged about 6,000 per c.mm. in normal animals, gradually increased during the experiment to 15,500 per c. mm., and, just before death, rather quickly fell to 4,500 per c.mm. The body temperature was always observed to fall gradually during the development of hematoporphyrin shock. In spite of the fact that the heat of the sunlight was often very intense, the rectal temperature often reached 28-30°C. just before death.

SUMMARY.

The results of these observations may be briefly summarized as follows:

Feeding of hematoporphyrin to white mice over long periods of time produced no apparent changes in these animals and had no effect upon their sensitivity to light.

Albino and slightly pigmented mice and rats injected with hematoporphyrin were protected from the rays of the sun by staining them a blue-black color with Verhoeff's hematoxylin. The dioxyphe-nyl-lanine (Dopa) reaction revealed no changes in the cutaneous pigment of animals injected with hematoporphyrin and exposed to sunlight, kept in the dark or diffused daylight. It was therefore assumed that the natural pigment of the skin plays only a physical rôle in protecting animals injected with hematoporphyrin from sunlight.

Exposure to sunlight of only the intestine and mesentery of a cat under ether anesthesia, which had been injected with hematoporphyrin, was followed by death of the animal.

Repeated injections into white mice of large amounts of blood from guinea pigs in hematoporphyrin shock failed to produce symptoms of hematoporphyrin shock. In a parabiosis experiment, one of a pair of white rats promptly developed characteristic symptoms and died

when injected with hematoporphyrin and exposed to sunlight, while the other animal, which was protected from light, but whose circulation had been demonstrated to connect freely with that of its partner, showed no changes during the entire procedure. It has, therefore, been impossible, so far, to demonstrate any substance present in the blood of animals in hematoporphyrin shock which is capable of reproducing this condition in other animals when introduced into the circulation.

Injection of hematoporphyrin followed by exposure of the entire animal to sunlight has been found to produce physiological changes in cats similar to those observed in traumatic shock. There promptly occurred a rapid fall of blood pressure to a very low level and marked lowering of body temperature. The venous blood was found to be poor in oxygen, rich in carbon dioxide and to show low carbon dioxide-combining power. The respiration, which first was accelerated, later on became deep and irregular. The reflexes and typical blood pressure responses to cutaneous and vagal stimulation could always be obtained until death.

Marked diminution of oxygen and increase of carbon dioxide content were found to occur in mixtures of blood and hematoporphyrin exposed *in vitro* to sunlight. These changes in the blood, identical with those occurring *in vivo* during hematoporphyrin shock, support Gaffron's views regarding the effect produced by the combined action of hematoporphyrin and light, but do not further elucidate the nature of the manner in which such alterations take place.

Unsuccessful attempts were made to produce, in both cats and dogs, physiological changes similar to those observed in hematoporphyrin shock by exposing only the blood flowing through a quartz glass cannula, connecting the femoral artery and vein, to strong arclight and sunlight. In another series of animals, which were first injected with hematoporphyrin, exposure of the circulating blood alone to arclight or sunlight did not produce hematoporphyrin shock, although the blood pressure did fall to an unusually low level in one instance.

No changes were found to occur in the amount of non-protein nitrogen, sugar or creatinine of the blood of animals in hematoporphyrin shock.

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RECIPROCAL EFFECTS OF CONCOMITANT INFECTIONS.

I. THE INFLUENCE OF VACCINIA ON THE REACTION TO INFECTION WITH EXPERIMENTAL SYPHILIS.

By LOUISE PEARCE, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

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The existence of two infectious diseases concurrently in the same individual is now well recognized as not infrequent, and it has been pointed out, among other interesting observations, that the symptoms of each disease may be more severe than when either occurs alone. Probably the infections which most often develop concomitantly or approximately so, are the acute infections of childhood, as for example, scarlet fever and diphtheria, or measles and diphtheria. In other infectious conditions, an intercurrent or complicating infection is a relatively common occurrence, and again, in such circumstances there may be an intensification of symptoms of one or both diseases, as for instance in tuberculosis and syphilis, or measles and tuberculosis. The unfavorable effect of influenza on certain patients with syphilis was shown during the epidemic of 1918 and 1919 in which there were many examples of an abrupt and serious change in a previously mild syphilis following an attack of influenza. On the other hand, a pre-existing disease may be favorably influenced for a longer or shorter time by an intercurrent infectious process, as for example, certain types of psychoses including general paresis, or even cases of malignant disease.

No satisfactory explanation of the various effects observed in these circumstances has been made, for the lack of accurate information regarding their precise nature has not permitted any but the most general statements. It is surprising, in view of the modification of symptoms which may occur and the significance which such alterations undoubtedly have from the standpoint of the host's reaction to disease, that the subject has received comparatively little experimental investigation.

Our interest in concomitant diseases arose first during our study of experimental syphilis and yaws, particularly with reference to superinoculation and crossed inoculation, and later, in connection with a transplantable malignant neoplasm of the rabbit which developed in a syphilitic animal. More recently our attention was again directed to this subject as a field for experimental investigation from observations of a strain of *Treponema pallidum* obtained from a rabbit found to be also infected with vaccine virus. It became evident that this virus was being transferred with the syphilitic inocula. The syphilis which developed in rabbits inoculated with this material was unusually mild, and this finding was wholly unexpected since this particular strain has been carried in rabbits for many years and is reported to be capable of inducing a severe disease. The possibility that the accompanying vaccinal infection contributed to these unforeseen results was naturally considered, but the acceptance of such an explanation obviously required experimental evidence obtained under properly controlled conditions. To this end, several series of experiments were undertaken in which was studied the influence of a vaccinal infection upon the reaction to syphilis induced by a strain of *T. pallidum* uncontaminated with vaccine virus. An extension of the investigation comprised experiments dealing with the reaction to *T. pallidum* of rabbits immune to vaccine virus.

The present paper contains the results of experiments in which one of the two commonly used routes of syphilitic inoculation was employed, namely, the intratesticular; the vaccinal inoculations were made intracutaneously on the side of the body. Subsequent papers contain the results of experiments in which *T. pallidum* and vaccine virus were inoculated in the same testicle, with observations on the syphilitic reaction of rabbits immune to vaccine virus and with the results of the intracutaneous route of both syphilitic and vaccinal inoculations. A summary of the results obtained has already appeared (1).

EXPERIMENTAL.

Materials and Method.

Three experiments are reported on the course of syphilitic infection in rabbits inoculated simultaneously with vaccine virus and with the Nichols strain of

T. pallidum. For comparison, other series were inoculated only with *T. pallidum*. The Noguchi strain of vaccine virus which was used was obtained from a fresh testicular lesion; its virulence was controlled by inoculation in normal rabbits using the intracutaneous and intratesticular routes as well as applications to scarified skin areas.

The experimental animals were inoculated intracutaneously (0.2 cc.) and on a scarified skin area (0.2 cc.) with vaccine virus and immediately thereafter with *T. pallidum* injected into the right testicle. The emulsions used were prepared from actively growing testicular lesions and contained from 1 to 3 spirochetes to the microscopic field; each animal received 0.2 cc.

The rabbits employed were young adult male animals approximately 8 months of age. They were placed in individual cages and were divided into 2 groups as nearly comparable as possible with respect to age, breed and weight. In each experiment, the control group, that is the group inoculated only with *T. pallidum*, comprised 10 rabbits; in the first and second experiments there were 5, and in the last, 10 animals in the vaccinated group.

The dates of inoculations were as follows: November 10, 1926, January 13 and February 14, 1927. The periods of observation were from 3 to 5 months, but for the purpose of comparing the results of the 3 experiments one with another, the data obtained during the first 3 months following inoculation have been used.

In these experiments special attention has been given to the time and frequency of occurrence and to the duration of successive phases of the reaction to syphilitic infection with a view to reducing the comparison of results to as quantitative a basis as possible. The particular conditions chosen for comparison were, (1) the incubation time of primary lesions, (2) the time and frequency of the occurrence of a critical edema in the inoculated testicle, (3) the time and frequency of occurrence of lesions in the uninoculated testicle (metastatic orchitis), (4) the time and frequency of occurrence of generalized lesions in the skin and mucous membranes, bones or eyes, (5) the number of foci affected by such lesions, (6) the proportion of animals that showed complete healing of all lesions during the 3 months observation period.

It should be noted, in discussing time relations of the various reactive phenomena, that there is a basic tendency toward the preservation of a uniform interval of time between the occurrence of successive reactions in syphilitic rabbits. With the Nichols strain of *T. pallidum* as carried in our laboratory, the reaction interval is approximately 2 weeks. In these experiments, as stated above, the Nichols strain was used, but the particular line from which the present substrain, as it may be termed, was derived had been transferred under somewhat different conditions from those employed with the parent strain. It was not known at the time this work was started, however, that the infection induced by this substrain would differ in any essential respect from that of the parent strain, but the results of these and other experiments have shown certain peculiar features of the syphilitic reaction, notably a delay in the development of a metastatic orchitis and an un-

usually early appearance of generalized lesions. These peculiarities in the behavior of the strain must be taken into account in analyzing the results obtained.

The methods employed for recording results require little explanation. The term "focal distribution" or "focal incidence" as applied to generalized lesions refers to the number of discrete foci at which lesions developed as determined by actual count. The figures for actual distribution are the mean values for those animals of a group that actually developed generalized lesions, while the figures for relative distribution give the results in terms of the entire group. This distinction is made in order to permit comparison of the extent of the lesions irrespective of the number of animals affected and at the same time to avoid any erroneous impression that might arise from the chance occurrence of an occasional case of severe syphilis in any group of animals.

Results.

The results of the experiments are recorded in Tables I, II and III and in Text-figs. 1 and 2. It will be noted that the figures given in

TABLE I.

Incidence of the Various Phenomena of the Syphilitic Infection and the Focal Distribution of Generalized Lesions.

Experiment	Animal group	No. of rabbits	Primary orchitis	Edema of inoculated testicle	Metastatic orchitis	Generalized lesions		
						Incidence	Focal distribution	Focal distribution
			<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>actual</i>	<i>relative</i>
I	C	10	100.0	60.0	100.0	100.0	9.8	9.8
	V V	5	100.0	80.0	100.0	100.0	22.2	22.2
II	C	10	100.0	50.0	90.0	70.0	7.0	4.9
	V V	5	100.0	40.0	80.0	100.0	14.4	14.4
III	C	10	100.0	70.0	90.0	90.0	8.4	7.6
	V V	10	100.0	44.4*	88.8	100.0	16.3	16.3
Mean values	C	30	100.0	60.0	93.3	86.7	8.6	7.4
	V V	20	100.0	52.6	89.5	100.0	17.4	17.4

C = controls; V V = animals inoculated with vaccine virus.

* One animal in this group which developed edema died shortly thereafter; its inclusion would bring the incidence of edema to 50.0 per cent.

Tables I and II and in the text-figures represent group values. In Text-fig. 1, the detailed results are presented graphically in 3 charts in order to show the entire sequence of events from the development of the first or primary lesions to the appearance of the last generalized lesions; the abscissæ represent the time after inoculation in days. These curves also illustrate an important feature of the syphilitic reaction, namely, that while these events occur successively, they also overlap each other to some extent. In order to simplify the reading of the charts, a division of the curves has been made at the 35th day

TABLE II.

Mean Time of Occurrence of the Various Phenomena of the Syphilitic Infection as Estimated in Days from the Date of Inoculation.

Experiment	Animal group	Primary orchitis	Edema of inoculated testicle	Metastatic orchitis	Generalized lesions		
					First	Last	Mean of all
		<i>days</i>	<i>days</i>	<i>days</i>	<i>days</i>	<i>days</i>	<i>days</i>
I	C	12.8	31.0	46.5	48.0	78.8	62.8
	V V	12.4	23.5	40.4	41.2	95.0	62.7
II	C	22.9	40.4	61.8	62.0	80.4	67.4
	V V	23.6	31.0	58.5	63.4	87.4	75.9
III	C	12.2	33.9	45.3	47.6	67.6	55.0
	V V	12.0	25.3	43.0	43.4	80.1	59.6
Mean values	C	15.8	34.7	51.0	51.6	75.1	61.0
	V V	15.7	25.7	45.9	48.1	88.6	64.0

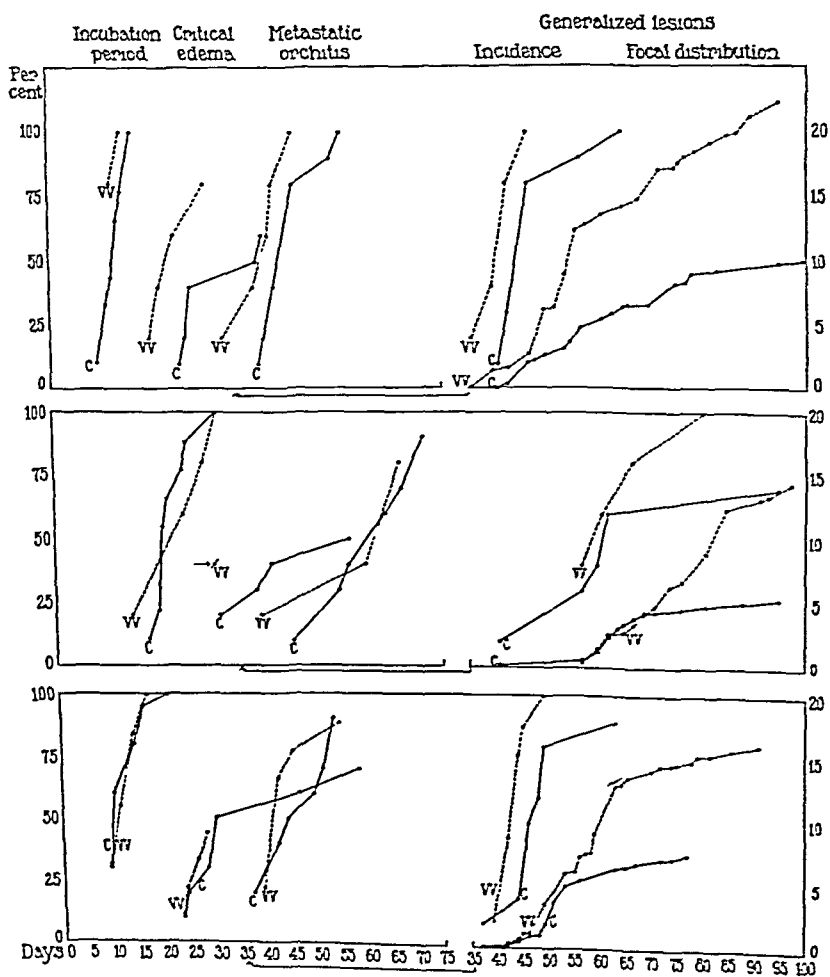
as indicated by arrows, and the curves representing the incidence and focal distribution rates of generalized lesions have been separated from the others. The time of appearance or the distribution of generalized lesions is shown by the curves in Text-fig. 2 in which a time interval of 4 days has been used. Table III contains the total number of generalized lesions. In the following discussion, mean values have been used for the most part since the results of the individual experiments are in essential agreement one with another.

TABLE III.

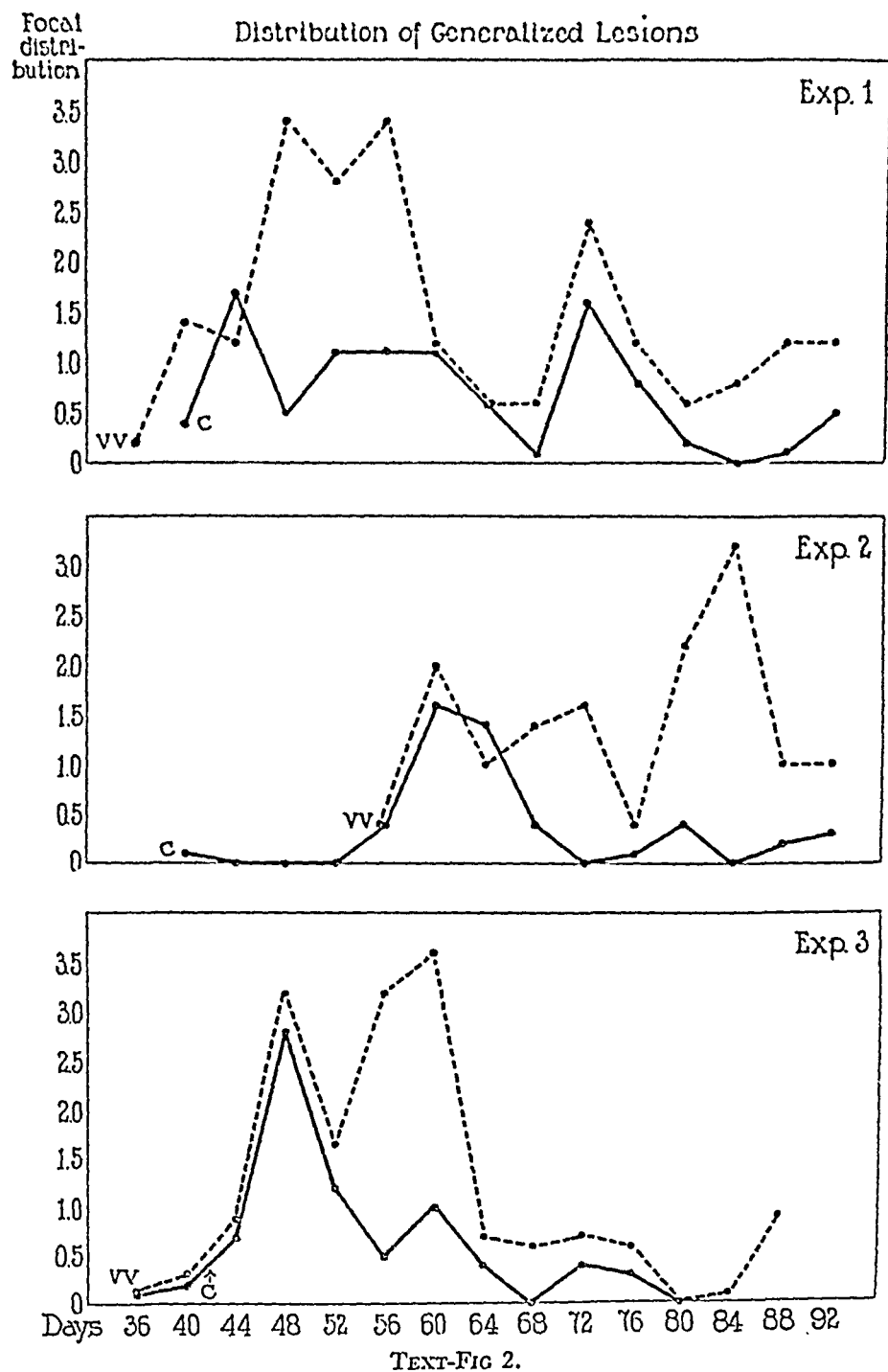
Time of Appearance of the Generalized Lesions as Estimated from the Date of Inoculation.

Time interval	Experiment I		Experiment II		Experiment III	
	No. of lesions		No. of lesions		No. of lesions	
	C (10 rabbits)	V V (5 rabbits)	C (10 rabbits)	V V (5 rabbits)	C (10 rabbits)	V V (9 rabbits)
<i>Days</i>						
35	0	1	0	0	0	0
37	0	0	0	0	1	1
39	0	6	0	0	0	0
41	1	0	1	0	2	3
43	3	1	0	0	3	4
45	0	0	0	0	4	4
47	19	6	0	0	1	0
49	3	17	0	0	9	20
51	0	1	0	0	18	9
53	6	13	0	0	12	13
55	5	16	0	0	5	13
57	11	1	4	2	0	3
59	0	0	7	5	0	14
61	7	6	9	6	0	0
63	4	0	7	0	10	34
65	6	3	3	1	1	5
67	0	3	4	3	3	0
69	1	0	3	0	0	5
71	0	0	1	7	3	4
73	0	11	0	8	1	1
75	16	3	1	0	0	1
77	8	2	0	2	3	1
79	0	2	0	10	0	4
81	0	3	4	11	0	0
83	2	0	0	0	0	1
85	0	3	0	17	0	0
87	0	1	2	0	0	2
89	1	6	0	4	0	0
91	5	6	3	6	0	5
Total	98	111	49	72	76	147

Influence of Concomitant Vaccinal Infection on Reaction to Syphilis



TEXT-FIG 1.



DISCUSSION.

The purpose of this investigation was to determine whether a concomitant vaccinal infection would influence the reaction to experimental syphilis. It should be kept in mind that in the experiments reported in the present paper, the inoculation of both viruses was made at the same time but at different sites—the vaccine virus intradermally on the side of the body and *T. pallidum* in one testicle. The results obtained have been analyzed in terms of various reactive phenomena in accordance with the general principles that govern the evolution of syphilitic infection (2, 3) and in the following discussion these phenomena are taken up in the order of their occurrence, so that the picture of the disease as it developed may be more readily visualized.

Incidence of Primary Lesions and Incubation Period.—All rabbits in these experiments developed primary lesions (Table I; Text-fig. 1), and there was practically no difference in the length of the mean incubation period between the vaccinated and control groups, that is 15.7 and 15.8 days (Table II). It will be noted that all incubation periods for the first and third experiments were essentially the same, 12.0, 12.2, 12.4 and 12.8 days, while those of the second were prolonged to 22.9 days in the case of the controls and to 23.6 days for the vaccinated group. As will be seen later, the magnitude of these values are indicative of the general character of the disease that prevailed, that is, it was much less severe in the second than in the first or third experiment.

It is evident from these findings that the concomitant intradermal inoculation of vaccine virus did not influence either the incidence of the primary syphilitic reaction or the time of its development as determined clinically by palpation.

Critical Edema.—While the occurrence of a critical edema in an inoculated testicle or in association with other syphilitic lesions is a variable phenomenon, it has an important significance as indicating an intense reaction; it usually marks the end of a local reaction, either temporary or final.

The mean values of the incidence of edema in the inoculated testicle were essentially the same for the controls and the vaccinated animals,

that is 60.0 and 52.6 per cent respectively, but the incidence in individual experiments was variable (Table I and Text-fig. 1). In the first experiment in which the disease was very severe there was a higher frequency in the vaccinated than in the control groups, but this order was reversed in the third experiment with a disease of somewhat less severity, while in the second in which a relatively mild syphilis prevailed, there was little difference between the 2 groups.

The time at which edema occurred, however, indicates very clearly the more prompt reaction of the vaccinated animals as shown by the mean values calculated from the time of inoculation, that is 28.6 for the vaccinated and 34.7 days for the control group (Table II). The results of individual experiments were similar, the difference in time between vaccinated and control groups being 7.5, 9.4 and 8.6 days respectively. In addition, it should be pointed out that there was a closer agreement in the time of development of edema among individual animals of each vaccinated group than among the controls as shown by the curves of Text-fig. 1. In the control groups, there were 2 rabbits each in the first and third and 1 in the second experiment in which an edema of the inoculated testicle was considerably delayed. And although the group and mean time values are obviously influenced by these particular animals, still their omission does not disturb the order of group values as shown by the following figures:

Experiment	"Corrected" controls		Vaccinated	
	No. of rabbits	Edema <i>days</i>	No. of rabbits	Edema <i>days</i>
I	4	26.3	4	23.5
II	4	36.3	2	31.0
III	5	26.8	4	25.3
Mean values	13	29.5	10	25.7

An idea of the progress of the reaction is also obtained by estimating the time of appearance of edema from the incubation period of the primary lesion instead of from the date of inoculation, as has been done in the table at top of following page.

From Incubation.

Experiment	All controls	"Corrected" controls	All vaccinated rabbits
	<i>days</i>	<i>days</i>	<i>days</i>
I	18.2	13.5	11.1
II	17.5	13.4	7.4
III	21.7	14.6	13.3
Mean values	18.9	13.7	10.0

A comparison of these values brings out very clearly the fact that there was a more prompt reaction to the syphilitic infection in the vaccinated than in the control rabbits irrespective of whether all controls are included in the comparison or only those animals in which the development of edema was not delayed.

As has already been mentioned, the usual time interval between the occurrence of successive reactions with highly virulent strains of *T. pallidum* is 2 weeks. The above table shows that with respect to the interval between the incubation period of the primary lesion and the critical edema, the majority of controls conformed to this value (13.7 days), while it was somewhat lengthened in the case of all controls (18.9 days). With the vaccinated animals, on the other hand, this interval was shortened to the unusually short time of 10.0 days.

The essential point to be remembered with respect to the phenomenon of critical edema is the earlier time in which this reaction occurred in the vaccinated groups as compared with the controls of these experiments, bearing in mind that this interval in the controls was not shorter than that usually seen in rabbits infected with the Nichols strain.

Metastatic Orchitis.—One of the most characteristic and regular phenomena of the syphilitic reaction is the occurrence of a metastatic orchitis in the uninoculated testicle which with a disease of well marked severity develops approximately 6 weeks after inoculation.

The incidence of a metastatic orchitis in the control and vaccinated groups of the present experiments was the same, as is shown in Table

I and Text-fig. 1. It occurred in all animals of both groups in the first experiment in which a disease of marked severity prevailed, while it was not detected in 1 animal each of the vaccinated and control groups of the second and third experiments in which the disease was less pronounced.

With respect to the time at which a metastatic orchitis developed, the vaccinated groups showed a more prompt response (Table II). The mean values reckoned from the day of inoculation were 51.0 days for the controls and 45.9 days for the vaccinated animals. These mean values are higher than those ordinarily seen with strains of high virulence, owing largely to the prolonged reaction time observed in the second experiment. But in this experiment as well as in the others, an orchitis of the uninoculated testicle occurred earlier in the vaccinated than in the control groups, the difference in time being 3.1 days in the second, 6.1 days in the first and 2.3 days in the third experiment. Moreover, as shown in Text-fig. 1 it uniformly occurred sooner in the vaccinated group than in the controls in the first experiment, and so too with most of the animals in the third experiment. In the second experiment, however, as might be expected with a mild disease, the lesions developed irregularly with no definitely defined group difference.

The results bearing on the development of a metastatic orchitis with respect to the time values of the previous reactions, that is to say, the incubation period of the primary lesion and the critical edema are shown by the following figures:

From the Incubation Period.

Experiment	Controls	Vaccinated rabbits
	<i>days</i>	<i>days</i>
I	33.3	28.0
II	38.9	34.9
III	33.1	31.0
Mean values	35.2	30.2

From the Critical Edema.

Experiment	Controls	"Corrected" controls	Vaccinated rabbits
	<i>days</i>	<i>days</i>	<i>days</i>
I	15.2	20.2	16.9
II	21.4	25.5	27.5
III	11.4	18.5	17.7
Mean values	16.3	21.7	20.3

With the Nichols strain, the interval between the development of a metastatic orchitis and the incubation period of the primary orchitis is usually from 4 to 5 weeks. In the case of the first and third vaccinated groups as well as with the mean value for the 3 groups this interval was actually or approximately 4 weeks, while the average intervals for the individual control groups as well as the mean value were approximately a week longer. Comparisons based upon the interval between the occurrence of the critical edema and the development of the metastatic orchitis are less satisfactory with group than with individual animals because of the variability in the incidence and time of occurrence of this phenomenon. In the present instance, if the comparison includes all control animals that developed edema, the interval is considerably shortened, owing to the delayed edema of 5 rabbits, and the resulting mean and individual group values are consequently smaller than the corresponding ones for the vaccinated animals. But with the omission of these 5 rabbits ("corrected" controls), this interval between edema and metastatic orchitis is slightly shorter in the vaccinated than in the control groups of the first and third experiments. It is slightly longer in the second experiment, a discrepancy which may be explained by the difference in the number of animals in the control (corrected) group which developed edema—that is 4 as contrasted with 2 in the vaccinated group. Such small differences, however, are of interest only in that they are in agreement with other results.

From the above analyses of the results obtained in connection with the incubation period of the metastatic orchitis with reference to the time of inoculation as well as from the time relations between its oc-

currence and that of preceding reactive phenomena, it is clear that the reaction to the syphilitic infection as measured by the phenomena of a metastatic orchitis was more prompt in rabbits which were vaccinated with vaccine virus coincidentally with the inoculation of *T. pallidum* than in control animals. It will be recalled that a similar result was obtained in connection with the preceding phenomenon of a critical edema.

Generalized Lesions.—The phenomenon of generalized lesions is the most indicative single basis for estimating the character of the reaction to syphilitic infection. The occurrence or non-occurrence of generalized lesions, the time of their appearance, and the duration of the period of active development, their number, extent, character and persistence are essential features of this phase of the disease which can be employed in analyzing its character. The disease exhibits a well recognized variability in all these respects, but it is usually comparatively constant under a given set of conditions.

In the present experiments, a large number of control animals developed generalized lesions, 86.7 per cent, but this was surpassed by the vaccinated groups with the unusually high incidence of 100.0 per cent (Table I). As has been mentioned before, the disease in the first experiment was very severe, in the second, comparatively mild, while in the third it was quite severe, and these differences are indicated by the incidence of generalized lesions in the control groups, namely, I, 100.0 per cent; II, 70.0 per cent; III, 90.0 per cent (Table I), but irrespective of these variations, generalized lesions occurred in every vaccinated rabbit.

In addition, an extremely large number of lesions developed in the vaccinated animals, the mean actual and relative distribution rates for the vaccinated groups being 17.4 as compared with control rates of 8.6 and 7.0 (Table I). Results of similar orders of magnitude were obtained in individual experiments. The extent of the differences between the vaccinated and control groups in this respect is better appreciated by estimating the focal distribution rates of the vaccinated groups in percentage terms of control values, as has been done in the table at top of following page.

Experiment	Actual rate of control values	Relative rate of control values
	<i>per cent</i>	<i>per cent</i>
I	+122.4	+122.4
II	+132.9	+230.6
III	+94.1	+114.5
Mean values	+102.3	+148.6

These figures demonstrate in a striking manner that the so called generalized phase of the syphilitic infection as measured by the actual number of lesions detected was much more pronounced in the animals which received an intracutaneous injection of vaccine virus at the time of intratesticular inoculation with *T. pallidum* than in the controls.

The time at which generalized lesions developed is another point of comparison in which the vaccinated rabbits differed from the controls. Reckoned from the day of inoculation, as has been done in the analyses summarized in Table II, the mean time of the appearance of the first generalized lesions in the vaccinated groups antedated that of the controls by 3.5 days, while the mean time of the last lesions to develop was 14.5 days later in the vaccinated than in the control animals. These differences as well as those obtaining in individual experiments are shown in the following table in which a minus or a plus sign indicates a shorter or a longer time than that for the controls.

Difference in Time of Appearance of Generalized Lesions of Vaccinated Groups as Compared with Controls.

Experiment	First lesion	Last lesion
	<i>days</i>	<i>days</i>
I	-6.8	+17.2
II	+1.4	+7.0
	(-2.1)*	
III	-4.2	+12.5
Mean values	-3.5 (-3.9)*	+13.5

* Values obtained by omitting one control animal with a single precociously early lesion.

Although the differences with respect to the last lesions are more striking than those for the first, still the first differences demonstrate the significant fact that the reaction time of the vaccinated groups in regard to another phenomenon of the syphilitic infection, that is to say, the development of generalized lesions, continued to be in advance of that of normal animals as was seen with the earlier phenomena of critical edema and metastatic orchitis. It should be mentioned that the corrected figure for the time difference in the second experiment is a fairer expression of the group picture than the uncorrected, as will be seen by referring to Text-fig. 1. That the severity of the disease prevailing in different experiments is an important factor in influencing the extent or range of the results obtained is well illustrated by the relative magnitudes of the values for the first and last lesions in the above table, the highest values being found in the first experiment in which the most severe disease prevailed, while the lowest occurred in the second with a comparatively mild disease.

From what has been said regarding the time of appearance of the first and last generalized lesions, it is obvious that the period of active development of this important phase of the disease was considerably longer in the case of the rabbits which received vaccine virus. The extent of these differences is strikingly brought out by the following comparison:

Period of Active Development of Generalized Lesions.

Experiments	Controls	Vaccinated	Increase of vaccinated groups	
	<i>days</i>	<i>days</i>	<i>days</i>	<i>per cent</i>
I	30.8	53.8	+23.0	or 74.8
II	18.4	24.0	+ 5.6	or 30.5
	(14.9)*		(+9.1)*	or (61.0)*
III	20.0	36.7	+16.7	or 83.5
Mean values	23.5	40.5	+17.0	or 72.3
	(23.1)*		(+17.4)*	or (75.3)*

* These values were obtained by omitting 1 animal with a precocious lesion, as explained in the foregoing text.

These figures show that in each experiment the period of active development of generalized lesions was much longer in the vaccinated

than in the control animals. The percentage increases in time of the first and third experiments, 74.8 and 83.5 per cent, are of the same order of magnitude as the mean value of 72.3 per cent. In the case of the second experiment, the increase of 30.5 per cent is considerably smaller, but the corrected figure of 61.0 per cent is comparable to the others; and as has already been mentioned, the corrected figure is more representative of the group as a whole.

In round numbers, the mean value of the duration of activity as regards generalized manifestations was 3 weeks in the case of the controls and 6 weeks in the case of the vaccinated animals. There was comparatively little difference between the 2 groups, however, in regard to the mean time of appearance for *all* generalized lesions reckoned from the day of inoculation, that is 61 and 64 days for the controls and vaccinated groups respectively (Table II); and a similar result is obtained if the interval is estimated from the incubation time of the primary orchitis. But if the comparison is made from the time of the reactive phenomenon immediately preceding the appearance of generalized lesions, namely, the development of a metastatic orchitis, the difference between the 2 groups is well shown. The results of these 2 analyses appear in the following tabulations:

From Primary Orchitis to Mean Time for All Generalized Lesions.

Experiment	Controls	Vaccinated
	<i>days</i>	<i>days</i>
I	50.0	50.3
II	44.5	52.1
III	42.8	47.6
Mean	45.8	50.0

From Metastatic Orchitis to Mean Time for All Generalized Lesions.

Experiment	Controls	Vaccinated
	<i>days</i>	<i>days</i>
I	16.3	22.3
II	5.6	17.2
III	9.7	16.6
Mean	10.5	18.7

Both these comparisons of time reactions show the existence of a longer interval between the date of the previous reactive phenomena and the mean time of appearance of all lesions in the case of the vaccinated animals as compared with the controls, but the difference is greater with respect to the phenomenon immediately preceding the development of generalized lesions, namely, the metastatic orchitis. From the various analyses of the results already presented, it is evident that this second comparison is more indicative of the general character of the disease prevailing in the 2 groups than in the first, and it illustrates one of the biological principles governing syphilitic infection, namely, that the character of any reaction is closely connected and, indeed, is particularly dependent upon the nature of the preceding reaction. In addition, the foregoing tables bring out the fact that, as regards these time relations, the vaccinated groups were more uniform than the controls, and this was to be expected under the conditions of relatively uniform disease severity prevailing in the vaccinated animals.

Neither of these comparisons involving the mean time of appearance for *all* generalized lesions, however, shows a marked divergence between control and vaccinated groups, and this is largely due to the small difference between these mean values. The differences that exist in the actual distribution of lesions in the vaccinated as compared with the control animals, however, are shown in the distribution curves of the times at which new lesions appeared (Text-fig. 2). A time interval of 4 days has been chosen in plotting these curves to obviate some of the irregularities associated with shorter periods, as shown in Table III which lists the total number of lesions in 2 day intervals. The relative rather than the actual number of lesions has been used because of the difference in number of animals in the groups of the first 2 experiments, so that in addition to the time distribution of lesions, the height of the curves gives an idea of the severity of the disease as measured by the numerical distribution of lesions in the order of their appearance.

Although the 3 sets of curves show certain dissimilar features due largely to the different character of the disease prevailing in individual experiments, those representing the vaccinated animals are in remarkable agreement as regards their general levels which are much higher

throughout than the control curves. For the most part, the fluctuations of the two curves in the first and third experiments, in which the disease was pronounced, occur in the same time interval, showing that in general the reaction of the vaccinated group had not been altered as regards the time of appearance of generalized lesions, but the much greater height of the curve for the vaccinated animals shows that the intensity of the reaction was profoundly affected as measured by the number of lesions which developed. There were three outstanding exceptions, however, to the synchronous time reaction of the vaccinated animals as compared with the controls. In the first experiment in which the most severe disease prevailed, the first generalized lesions appeared earlier in the vaccinated than in the control animals. In the second experiment many lesions developed in the vaccinated rabbits during the last 4 weeks of the observation period, while there were extremely few among the controls, and in the third, lesions were developing in the vaccinated animals when the period of observation was discontinued, although no lesions had appeared in the controls for the preceding 2 weeks.

Without analyzing these curves in detail, it is apparent that with a syphilitic disease of varying degrees of severity, it has been possible to increase the numerical distribution of generalized lesions and to prolong the period of active development of lesions over and beyond that of normal animals by means of an intracutaneous inoculation with vaccine virus made at the time of an intratesticular inoculation with *T. pallidum*. The general schedule of the times at which lesions first appeared, however, was not appreciably disturbed except in the experiment in which the disease of the control animals was mild, and this exception should be especially noted. The two curves of the second experiment are similar in their first portion, the peak of both occurring in the 60 day interval. The control curve then falls to the base line (72nd day) and practically continues there, while there are two high peaks in the vaccinated curve occurring in the 72nd and 84th day intervals, and at no time does it reach the base line. Furthermore, the second of these two peaks is higher than the first, showing that a greater number of lesions first appeared late in the course of the disease. Such an occurrence is unusual and indicates that earlier reactions were not sufficient to prevent the development of additional

and numerous late lesions; in the case of the control animals, as shown by the curve, an apparently similar early reaction was sufficient. In the third experiment, the second peak of the vaccinated curve closely follows the first, and apparently the reaction associated with such a shower of lesions was sufficient to prevent the later development of numerous lesions, although it should be noted that at its end, the curve rises abruptly to an appreciable height indicating that a number of generalized lesions were continuing to appear. In the first experiment, the curve of the vaccinated animals is a marked exaggeration of the control curve. It will be noted that the peak of the control curve in the 72nd day interval follows a long period (from the 40th day) in which many generalized lesions developed continuously at a fairly uniform rate and that there was not an early and relatively short period of marked eruptive activity which ordinarily would not be followed by successive periods of lesion development.

Recovery.—The last phase of the syphilitic reaction to be considered is that of complete resolution and healing of all lesions within the 3 months period of observation. Under ordinary circumstances, certain rabbits show no lesions by this time; in others residual lesions, usually of the genitalia, remain, while some animals continue to show active generalized manifestations. In the first and second experiments, the resolution of lesions was delayed, owing in one instance to the severity of the infection, and in the other to the late development of lesions and the consequent prolongation of the time of resolution and healing as reckoned from the day of inoculation. The condition of the lesions at the end of the observation period, therefore, has been described as follows: healed, not healed and active. The comparison of the vaccinated and control animals upon this basis gives the results, expressed in per cent, as shown on following page.

These figures show that the vaccinated animals differed markedly from the controls in the final phase of the syphilitic reaction, and furthermore, that the character of the difference was in accord with what was observed in earlier phases of the reaction. In the great majority of vaccinated animals, 89.5 per cent, the reaction to the syphilitic infection had not ceased at the end of the observation period, for lesions not only continued to be active, but as has already been mentioned, fresh lesions were developing in many animals. On the

other hand, only 26.7 per cent of the controls showed a comparable state of lesion activity, the majority of the animals having entered upon the phase of resolution.

Final Condition of the Lesions.

Experiment		Healed	Not healed	Active
		<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
I	Controls	10.0	50.0	40.0
	Vaccinated			100.0
II	Controls		60.0	40.0
	Vaccinated		20.0	80.0
III	Controls		100.0	
	Vaccinated		11.2	88.8
Mean values	Controls	3.3	70.0	26.7
	Vaccinated		10.5	89.5

At this point, something should be said of the general character of the lesions. It is well recognized that in a group of 5 or 10 rabbits, there is an individual animal variation as regards size, consistency and destructiveness of both genital and generalized lesions, which features are, of course, associated with the duration and degree of active development and persistence of lesions. In the present experiment, variations in these respects occurred in individual rabbits of both the control and the vaccinated groups, but in general, it may be said that both the genital and generalized lesions of the vaccinated animals were larger, more destructive and persisted longer than those of the controls. This difference was particularly striking in the case of scrotal chancres, and of bone and periosteal granulomata.

The disease which was observed in this investigation presented certain peculiar and uncommon features, especially of time reactions and of lesion type, which should be referred to, as they must be kept in mind in appraising the experimental results obtained. For example, the development of the metastatic orchitis was delayed while generalized lesions appeared unusually early, so that the two reactions occurred within a few days of each other instead of being separated by the usual interval of approximately 2 weeks. As a matter of fact,

in approximately one half of the total number of both the control and vaccinated animals, the appearance of generalized lesions preceded the detection of the metastatic orchitis by a few days, or both reactions were observed to occur on the same day as is shown in the following table:

Appearance of Generalized Lesions in Relation to Metastatic Orchitis.

Controls	Earlier	Same day	Total
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
I	40.0	20.0	60.0
II	30.0	10.0	40.0
III	40.0	10.0	50.0
Mean values	36.7	13.3	50.0
Vaccinated			
I	20.0	20.0	40.0
II	20.0		20.0
III	33.3	33.3	66.7
Mean values	31.6	21.1	52.7

These figures, as well as the time intervals between the development of the metastatic orchitis and the appearance of the first generalized lesions (Table II) indicate that an intracutaneous inoculation of vaccine virus coincident with the intratesticular inoculation of *T. pallidum* did not affect this peculiar feature of the disease.

The infection was further characterized by an unusually high preponderance of bone lesions and a relatively low number of skin lesions. Up to a certain point, such a distribution is to be expected, for numerous and severe cutaneous manifestations do not usually develop in animals in which previous bone involvement has been extensive. But this relationship does not obtain in cases of very severe or malignant syphilis which are characterized by numerous extensive and persistent lesions of both bone and skin and frequently of other tissues as well. In the first experiment, there were many instances of syphilis which could be called malignant judging from the character of genital and bone lesions, and in the second, there were several which approached this state, but nevertheless, there were comparatively few cutaneous manifestations, other than those of the

scrota which are not usually included among generalized or secondary lesions. This aspect of the infection is illustrated by the following table:

Relation of Skin and Bone Lesions.

Experiment		Total Relative No.	Bone Relative No.	Skin	
				Relative No.	Per cent of total
I	Controls	9.8	9.1	0.7 or	7.14
	Vaccinated	22.2	20.0	2.2 or	9.09
II	Controls	4.9	3.6	1.3 or	26.53
	Vaccinated	14.4	14.0	0.4 or	2.77
III	Controls	7.6	7.5	0.1 or	1.32
	Vaccinated	16.3	15.6	0.7 or	4.29
Mean values	Controls	7.4	6.7	0.7 or	9.44
	Vaccinated	17.4	16.4	1.0 or	5.75

The relatively large proportion of skin lesions in the control animals of the second experiment is apparently out of harmony with the findings in both groups of the first and third series. However, if the values for individual experiments are tabulated in the order of the severity of disease, the situation becomes somewhat clearer:

Experiment	Controls	Vaccinated
	<i>per cent</i>	<i>per cent</i>
I	7.14	9.09
III	1.32	4.29
II	26.53	2.77

This arrangement shows that for all vaccinated groups and for the first and third control groups, the proportion of skin to total secondary lesions follows a consistent numerical relation as regards the different levels of disease severity in the 3 experiments, and furthermore, that in the second experiment in which the infection was less marked than in the others, the proportion of skin to total lesions was not disturbed in the vaccinated animals. In the second control group, on the other hand, there was a relatively large number of cutaneous

manifestations but these were preceded by comparatively few bone lesions, a finding which was absent in the corresponding vaccinated group as is shown in the following table:

Relative Number of Bone Lesions.

Experiment	Controls	Vaccinated
I	9.1	20.0
III	7.5	15.6
II	3.6	14.0

It is evident from these figures that the numerical relationships of bone and skin lesions in the second vaccinated and control groups were in harmony with what is known of this phase of syphilitic infection, and the seeming contradiction of the second vaccinated group with respect to the number of skin lesions as compared with the corresponding control group is in reality in accord with the preservation of disease type and severity on the part of the vaccinated animals.

Still other evidence of the influence induced by vaccinal inoculation upon the course of the syphilitic disease is furnished by the distribution curves of generalized lesions (Text-fig. 2). The shape of the curves representing the control groups illustrate the general relations that obtain under ordinary conditions with respect to the numerical distribution of secondary lesions, that is, the early development of numerous lesions is not usually followed by the successive appearance of many others (see curves of Experiments II and III), while severe syphilis is characterized by the continued appearance of lesions for a prolonged period, a fact exemplified in the control group of the first experiment. The curves illustrating the distribution of lesions in all vaccinated groups show that this relationship was interfered with, for despite the large number of lesions that appeared early in the period of generalized manifestations, numerous lesions continued to develop in these animals.

It is evident from the results obtained in these experiments that intracutaneous inoculation with vaccine virus at the time of intratesticular inoculation of *T. pallidum* was associated with a profound disturbance in the syphilitic reaction, particularly as regards the rela-

tive frequency of occurrence of various reactive phenomena, the altered time relationships of these manifestations and in the relative failure on the part of one phenomenon to prevent or delay the development of successive manifestations. As measured by the various criteria selected for comparison, the efficiency of reaction of the vaccinated animals was unquestionably lowered with the result that the infection pursued a severe and uninterrupted course.

The coexistence of two or even three and four various infections has been attributed to an "increased susceptibility" on the part of the patient consequent upon the severity of the primary disease and in attempts to explain the gravity of these cases, the rapidity with which the diseases follow one another has been frequently invoked. In the present experiments, the syphilitic and vaccinal inoculations were made simultaneously. It must be remembered though that the reaction to vaccine virus develops more promptly than the syphilitic reaction as judged from gross manifestations although it is known that changes do occur at the site of inoculation and probably elsewhere within a few days after the injection of *T. pallidum*.

The present results indicate, nevertheless, that vaccination did not bring about a state of "increased susceptibility" to the syphilitic infection, if one means by this term an increased liability to infection as indicated by a shortening of the incubation period of the primary lesion. But the findings amply demonstrate that it did markedly affect the syphilitic reaction, and since the effect was evident not only early but late in the disease, it is evident that the disorganization of the reaction was profound. The fact that it was evident for so long a time, as well as certain peculiar changes in the reaction such as the shortened time interval between successive phenomena, the marked increase in the number of secondary lesions and their general character suggest that the general alteration was of the nature of a decreased resistance on the part of the animals.

From knowledge already available, it is clear that the syphilitic reaction involves many factors that are essentially expressions of functional activity which, in some instances at least, appear to be related to changes in physical constitution. It is not unlikely that the differences brought out in the present experiments are the result of similar changes.

In conclusion, it may be pointed out that the study of experimental coexistent or concomitant infections is of value as a means of investigating the general problem of susceptibility and resistance to disease. Hitherto, this study has been confined almost entirely to individual diseases.

SUMMARY.

Experiments are reported in which was studied the effect of a concomitant infection, vaccinia, upon the syphilitic reaction of rabbits. Vaccine virus was inoculated intracutaneously on the side of the body at the time of intratesticular inoculation with *Treponema pallidum*.

The results showed clearly that the vaccination caused a profound disturbance in the syphilitic reaction, the ensuing syphilis being extremely severe. From an analysis of various features of the reaction, it appeared that the factor of host resistance was primarily concerned in the effects observed.

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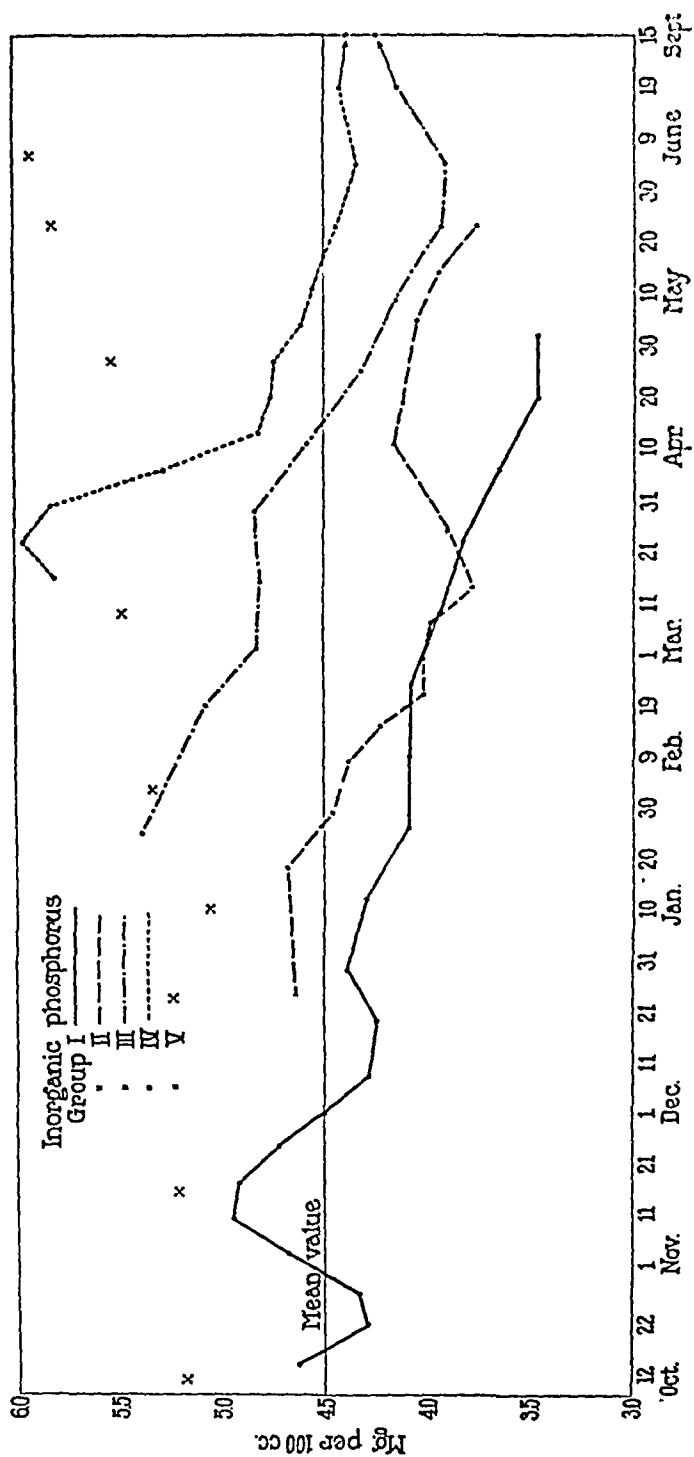
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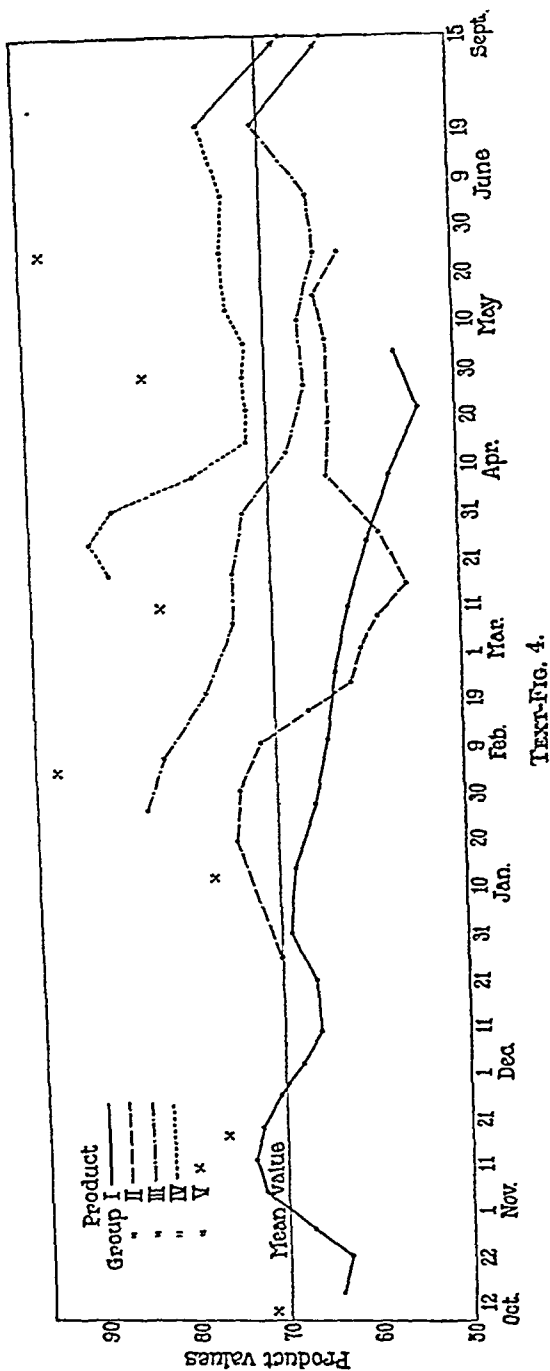
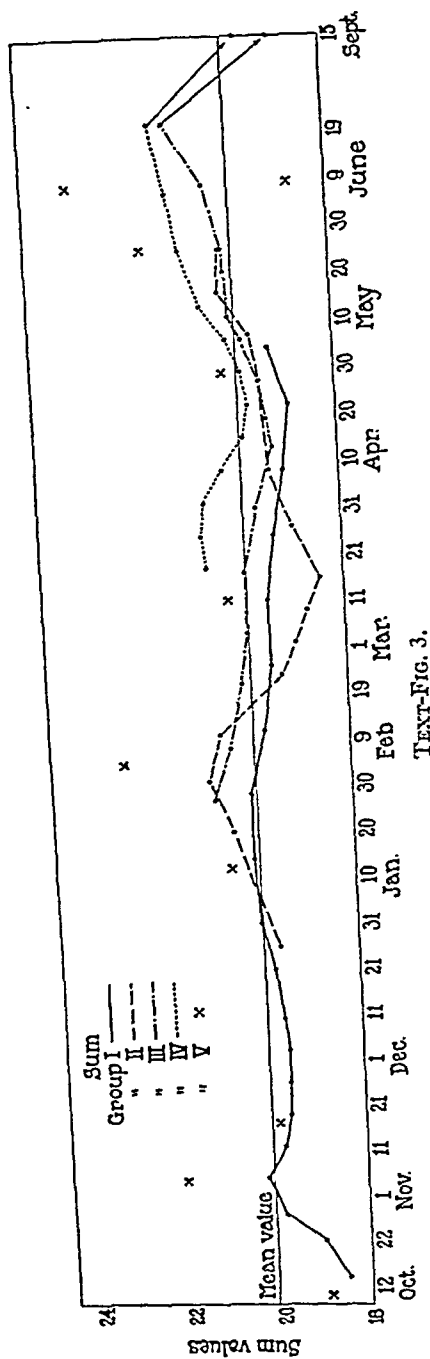
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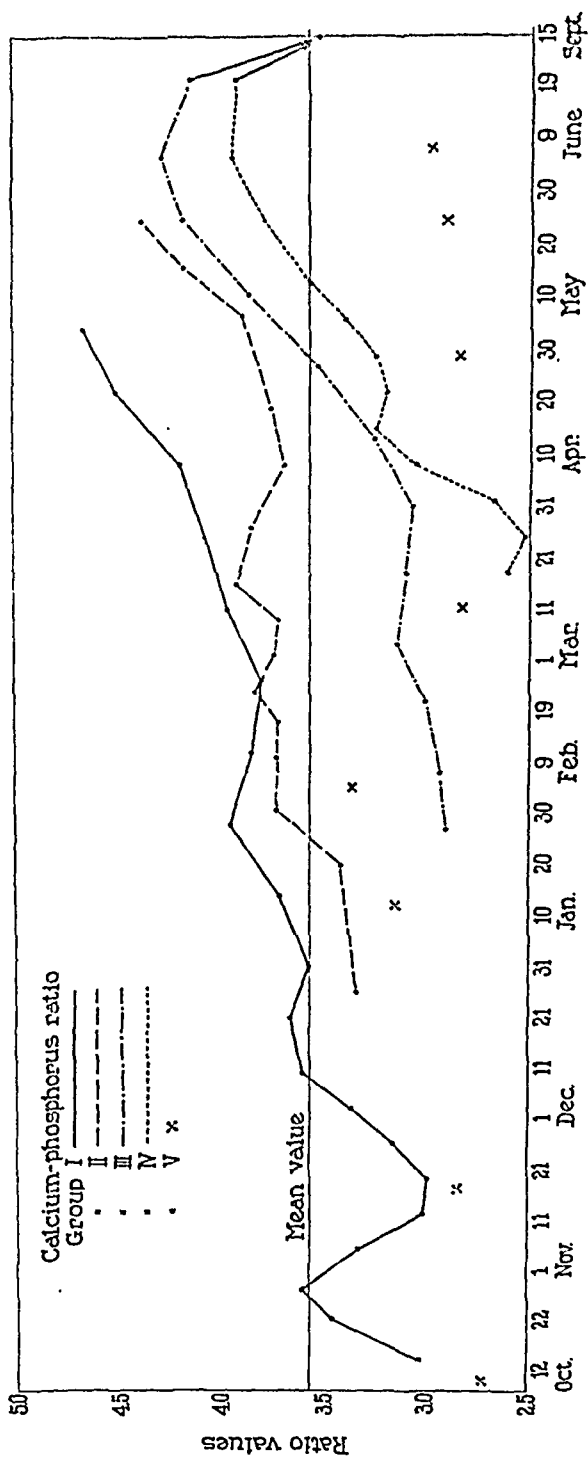
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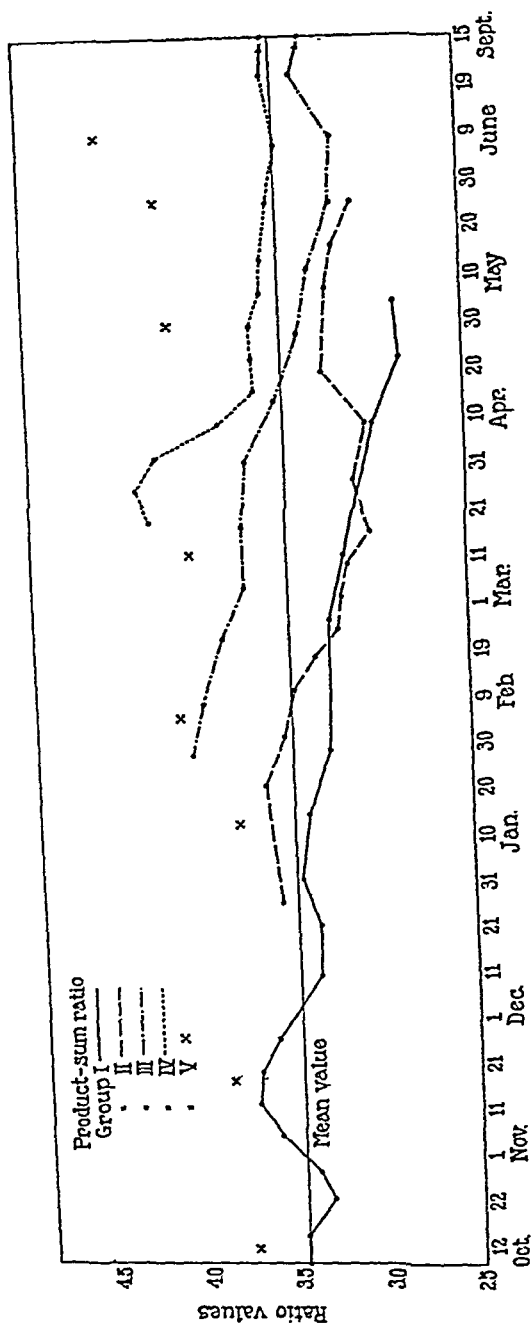


TEXT-FIG. 2.

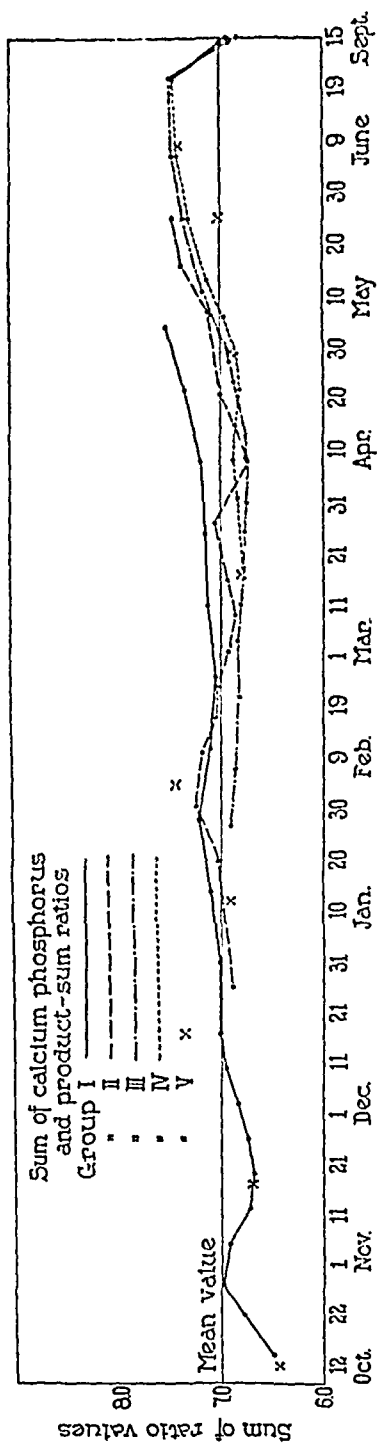




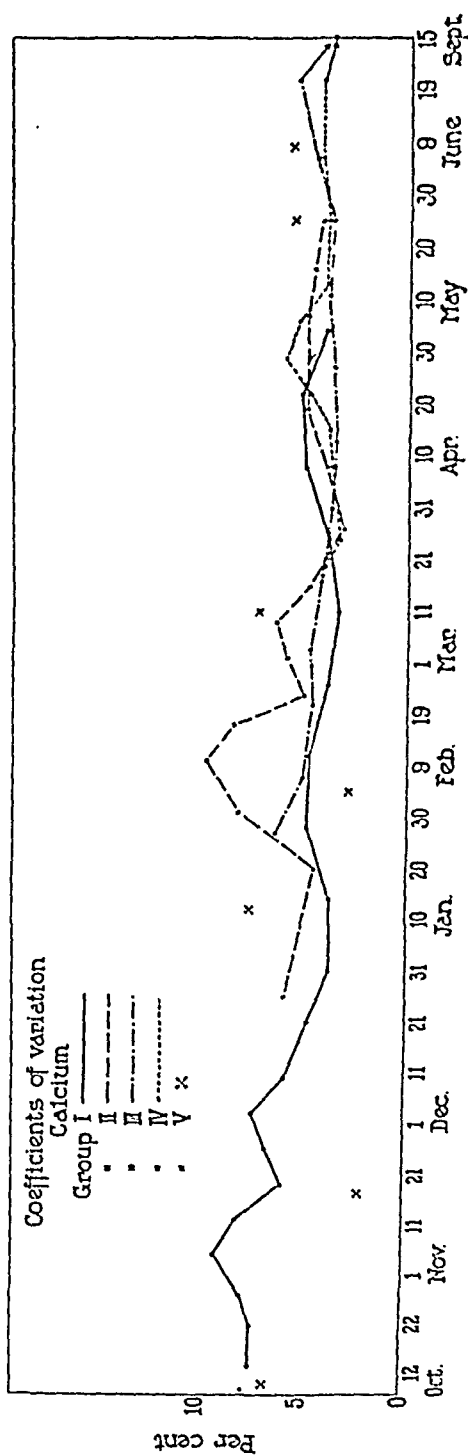
TEXT-FIG. 5.



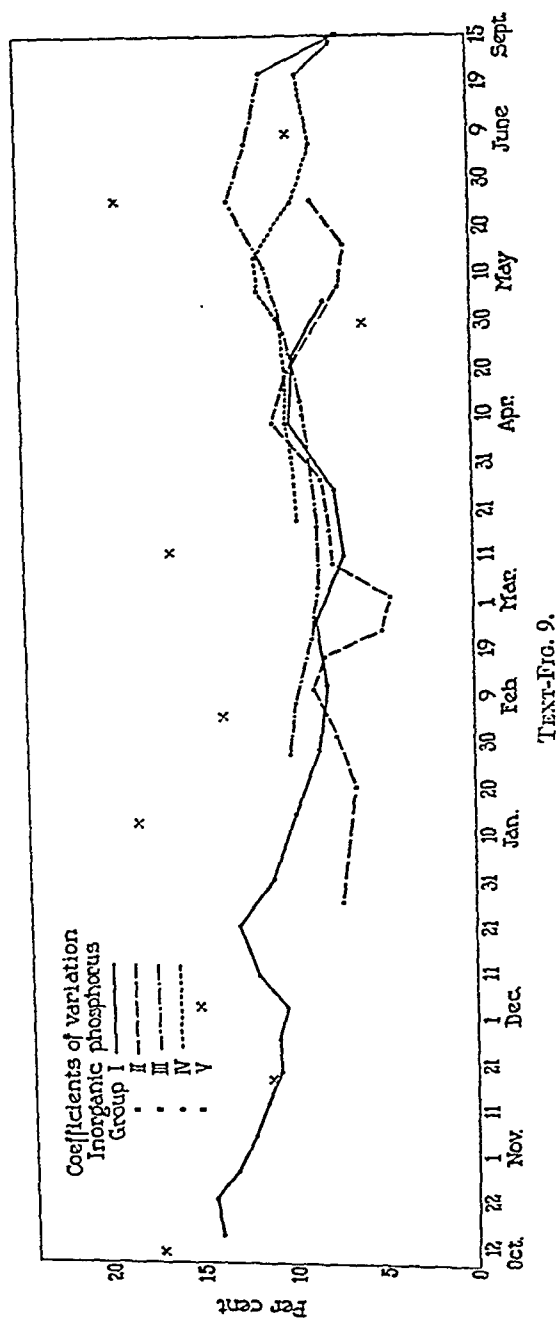
TEXT-FIG. 6.



TEXT-FIG. 7.



TEXT-FIG. 8.



DISCUSSION AND CONCLUSIONS.

An examination of the figures given in Tables I to VI and the curves in Text-figs. 1 to 9 shows at once that both the absolute values for calcium and inorganic phosphorus and the relative amounts of these substances exhibited an interesting series of variations in all of the animals studied. The changes observed in different groups of animals were not identical, but there were certain general features that were common to all, as may be seen by comparing the curves in Text-figs. 1 to 9. The course of events is more difficult to follow from the tabulated results.

In practically all instances, there are variations of two types. One of these is progressive in character and represents a gradual change of values from a lower to a higher, or from a higher to a lower level. This gradual change of level is accompanied in some instances by a clearly defined series of periodic variations, the positive and negative phases of which extend over periods of several weeks or months. In other cases, clearly defined periodic increases are absent, but the gradual change in level is marked by periods of stabilization at a given level, or by a temporary increase or decrease in the rate of change. The tabulated results show, however, that neither of these conditions represents a continuous movement in a given direction, but that the periodic variation is itself produced by a succession of pendulum-like fluctuations. Moreover, it will be seen that, to some extent, the progressive increase or decrease in values is in reality a long period variation as the high or low levels are not maintained indefinitely. This is particularly true of calcium as may be seen by reference to the tables and text-figures for Groups III and IV (Tables III and IV and Text-fig. 1).

From October until July, the general trend of calcium values is slightly upward (Tables I to V and Text-fig. 1). The outstanding feature of the condition shown by blood calcium is, however, the occurrence of a series of periodic increases and decreases. From October to December, 1926, consecutive observations were made on only one group of animals (Group I). These showed a gradual increase in calcium which reached a maximum about the last of October or the first of November (Table I and Text-fig. 1). This increase was fol-

lowed by a decrease which was succeeded in turn by a gradual and prolonged rise, terminating in a second maximum toward the end of January, 1927. A gradual fall in calcium then began and continued well into April. There was a suggestion of a secondary rise at the end of February and the first of March, but the third definite period of increase did not begin until the end of April or the first of May.

The variations in calcium shown by other groups of animals differed mainly with respect to the general level and the exact time of occurrence of maximum and minimum values. The conditions shown by Groups III and IV compare favorably with those of Group I (Tables III and IV and Text-fig. 1). The results for Groups III and IV are of especial interest in that they show that the period of increasing calcium which began in April or May continued through June, reaching a very high value on July 1. Unfortunately, no observations could be made on these animals during July and August, but by the middle of September, their calcium had again fallen to a point slightly below the mean normal level (15.6 mg.) or to values comparable with those for the preceding April and early May.

The interesting feature of the results for Group II is the extent of the variations shown (Table II and Text-fig. 1). The January-February maximum was considerably higher than that of Group I, while the March minimum was much lower. Moreover, the succeeding rise began while the calcium in the blood of other animals was still falling and well in advance of the rise in other groups.

Comparing the results for consecutive observations on animals caged in the laboratory (Groups I to IV) with those for animals examined as they were received from the dealer (Group V), it will be seen that, while the two sets of results agree with respect to the form of the variations, there is an interesting difference in the magnitude of the variations shown. In recently acquired animals, maximum values tended to be higher and minimum values lower, which suggests that the calcium content of the blood is subject to greater variation among animals living out of doors than among those caged in the laboratory.

The smoothed curves give the impression of a continuous or uninterrupted movement in a given direction which, with due allowance for technical inaccuracies and chance errors, is probably incorrect. A critical examination of the tabulated results will show that, in all prob-

ability, the prolonged rise and fall was accomplished by a series of short pendulum-like fluctuations with the general level rising or falling, as the case might be. This applies not only to calcium, but to all other values.

The situation presented by inorganic phosphorus is distinctly different from that of calcium (Tables I to V and Text-fig. 2). The striking feature of the results obtained for animals living under laboratory conditions is a marked and progressive decrease in inorganic phosphorus (Text-fig. 2); this is in sharp contrast with results for animals examined as they were received from the dealer. Available data for recently acquired animals show little or no change in inorganic phosphorus until January or February. There is a suggestion of a decrease in January and of a beginning rise in February. This rise was, however, comparatively slight and virtually ceased during March and April, to be resumed and continued through May and June.

Among the animals living under laboratory conditions, there are several features of the results that appear to be significant. Of these, the progressive decrease in inorganic phosphorus is most evident. It will be noted also that the results for each group of animals occupy a different level with comparatively little overlapping, and that the levels of successive groups rise in somewhat the same manner as the results for recently acquired animals examined at corresponding times (Text-fig. 2). Moreover, while all animals show a decrease in inorganic phosphorus, it will be seen that both the rate and extent of the decrease varied in different groups, and that in no case did the reduction proceed uninterrupted or at a uniform rate. In fact, each of the 4 groups of animals showed one or more periods during which the decrease in phosphorus ceased or gave place to an actual increase. This may be brought out by an analysis of the course of events in a given group of animals.

During November, the animals of Group I showed an abrupt increase in inorganic phosphorus extending over a period of 2 to 3 weeks with a return to the previous level about the first of December. This change coincided with a decrease and a subsequent rise in calcium. For 3 months thereafter, the decrease in phosphorus was comparatively slight, but during March and April it diminished at a more rapid rate, reaching the lowest level about the first of May. The last observation

on these animals gave a value that was higher than any that had been obtained since February which suggests that the phosphorus was again on the point of increase.

The distinctive feature of the results obtained for Group II is the marked decrease in inorganic phosphorus between January 5 and March 16, and the subsequent increase during March and April. In this instance, the change in inorganic phosphorus coincided with like changes in the calcium content of the blood.

The results for Groups III and IV show conditions that are again different. In the case of Group III, there was a reduction in inorganic phosphorus during the first 6 or 7 weeks; this was followed by an increase during March (Table III) and a second decrease which continued into June and was succeeded by a slight rise toward the end of June and the first of July. The striking feature of the change noted in Group IV is the marked reduction in inorganic phosphorus during the first 2 weeks of April. From this point on, the results compare favorably with those for Group III.

Considered as a whole, the results for inorganic phosphorus are somewhat confusing, due to the lack of agreement between recently acquired stocks and animals living under laboratory conditions, as well as to differences in the behavior of animals placed under observation at different times. Still, there is considerable evidence to show that inorganic phosphorus is subject to the action of influences which tend to raise or lower the level, as in the case of calcium, but these influences do not operate in precisely the same manner on animals confined in the laboratory and on those living out of doors. It is important to note that the change from outdoor to indoor life appears to be sufficient in most cases to initiate a decrease in the phosphorus content of the blood. In some instances, the immediate effect may be very slight, or even in the opposite direction, due perhaps to the fact that indoor conditions do not differ materially from those to which the animal has been accustomed (Group I); but, in other instances, the decrease in phosphorus may be marked (Group IV). Under such circumstances, the action of factors tending to increase the inorganic phosphorus of the blood may be completely obscured or accomplish no more than a reduction in the rate of decrease or a temporary stab-

ilization at a given level, such as occurred at several points in these experiments.

While it is reasonably certain that periodic variations in the trend of inorganic phosphorus occurred, definite movements can not be traced with the same degree of certainty as in the case of calcium. The indications are that there was a tendency toward lower values for animals of a given age, during the late fall and early winter months which was succeeded by a rise in January or February. The condition that prevailed during March and April is even more obscure, as some animals showed an accentuation of the decrease in inorganic phosphorus while others maintained a fairly high level. During May the trend was downward, and the final rise did not begin until June.

Applying the same principles of analyses to the values obtained for the sum and the product of calcium and inorganic phosphorus (Table VI and Text-figs. 3 and 4), it will be seen that, in general, these values reflect the variations shown by calcium and phosphorus respectively. This is particularly true of the sum, but the product shows a series of variations that is more clearly defined than those of phosphorus. In this case, the results obtained for recently acquired animals are of especial interest as they show a high value for February followed by much lower values for March and April and high values for May and June. In this respect, the results are in close agreement with those for calcium and for the sum of calcium and phosphorus.

The results for the ratio of calcium to inorganic phosphorus (Table VI and Text-fig. 5) disclose a condition that is almost the reverse of that shown by inorganic phosphorus. The general trend of values for all animals caged in the laboratory is upward, but variations in the absolute amounts of both calcium and phosphorus are reflected in these values.

Calcium-phosphorus ratio values for recently acquired animals show a gradual increase from October to February with a decided drop during March and April and a second slight rise during May and June. Animals in the laboratory show essentially the same conditions, if we disregard minor fluctuations. The chief difference between the two sets of values is that animals under continuous observation do not show a decided reduction in the ratio values for March and April, but a slight reduction or stabilization which is followed by a very

marked increase when the curve again turns upward. From observations on 2 groups of animals (Groups III and IV), it was found that the high values attained during this period of increase were eventually succeeded by a decrease. It is thus seen that, despite the progressive decrease in inorganic phosphorus, there is evidence of the occurrence of distinct periodic variations in the calcium-phosphorus equilibrium.

As a rule, the ratio of the product to the sum shows an inverse relation to the calcium-phosphorus ratio, and the sum or mean of the two values varies within very narrow limits or is virtually constant for a given set of conditions (1). An interesting exception to this rule is shown by the group of recently acquired animals (Table VI and Text-figs. 6 and 7). In this instance, it will be seen that the relation between values for the product-sum ratio and the calcium-phosphorus ratio is direct and not inverse. But, despite this difference, the quantitative relations are in close agreement with values obtained by repeated examination of given groups of animals, and these results give further evidence of the same series of periodic variations as was found by analysis of other values (Text-fig. 7).

The series of variations described above are, on the whole, so clearly shown by the curves that it would hardly seem necessary to resort to detailed statistical methods to determine whether variations of the order indicated represent actual differences in values or are within the range of probable variation due to random sampling. It is obvious that when a given change occurs gradually and by a series of pendulum-like swings, as in the present instance, nothing of importance can be learned by a comparison of successive values unless each value is based on a large series of observations. On the other hand, something may be gained by comparing values for given periods which represent a general trend or opposite phases of a supposed variation. A few comparisons of this kind will serve to indicate the probable significance of the results as a whole.

As the first example, we may compare the results obtained for calcium on Group I from November 12 to December 3 inclusive with those of December 17 to January 28 (Table I and Text-fig. 1). The mean values of all observations for these periods are $14.7 \pm .128$ and $15.9 \pm .099$ mg. respectively. As the difference of $1.2 \pm .16$ mg. is 7.5

times its probable error, it is certain that the difference between the values obtained for the two periods is significant.

There are even greater differences between results obtained for other groups during March and April, which in general represented a period of low calcium, and May, June, and July when the calcium rose to a much higher level. In the case of Group III, the mean value of all determinations from March 4 to April 27 inclusive is $15.0 \pm .062$ mg. and for May 11 to July 1, $16.7 \pm .082$ mg. which gives a difference of $1.7 \pm .10$. This difference is 17 times its probable error. The results for Group IV, comparing values for March 11 to April 1, and May 25 to July 1, are $15.1 \pm .068$ and $17.1 \pm .073$ mg. with a difference of $2.0 \pm .10$, which is 20 times its probable error. The March and April values for Group V give a mean of $15.0 \pm .104$ while the mean for May and June is $16.8 \pm .180$. In this case the difference of $1.8 \pm .21$ is 8.57 times the probable error.

Added significance is given to these results by the close agreement of the values obtained for different groups of animals at corresponding periods, as shown by the following tabulation of calcium values:

Group	Period of low values	Period of high values
III	$15.0 \pm .062$	$16.7 \pm .082$
IV	$15.1 \pm .068$	$17.1 \pm .073$
V	$15.0 \pm .104$	$16.8 \pm .180$

Less can be gained by a statistical analysis of the results for inorganic phosphorus. The progressive reduction in inorganic phosphorus among animals living under laboratory conditions is so evident that no further proof of the validity of this change is necessary. There are, however, a few instances in which a presumptive increase in inorganic phosphorus occurred in the course of the general downward movement, and the data bearing on these changes may be examined more critically. One of the best illustrations of a change of this kind is furnished by the increase in inorganic phosphorus which occurred in the animals of Group I during the middle of November. The observations for October 21 and 29 give a mean of $4.19 \pm .099$ mg. and those of November 12 and 19, $5.00 \pm .078$ mg. This difference is 6.43 times its probable error ($.81 \pm .126$) and hence is definitely significant.

An analogous increase in inorganic phosphorus occurred in the animals of Group II between April 6 and May 11. The mean value for this period was $4.13 \pm .075$ mg. as compared with a mean of $3.88 \pm .056$ for March. As this difference ($0.25 \pm .09$) is only 2.78 times its probable error, it may not be significant, but probably is.

A comparison of the values obtained for Group III on May 25 and June 8 with those for June 17 and July 1 gives a difference ($0.48 \pm .10$) that is 4.8 times its probable error, and the October and November results for Group V compared with values for May and June give a difference ($0.66 \pm .18$) that is 3.66 times the probable error. It is evident, therefore, that even in the case of inorganic phosphorus, there were instances in which a significant reversal of the dominant trend occurred.

Statistical comparisons of the results obtained for various measures of the relation between calcium and phosphorus have not been worked out in detail as it is evident that essentially the same situation obtains as in the case of calcium and phosphorus.

In this connection, however, some reference may be made to the degree of variation shown by the results obtained for individual animals at any given time as indicated by the coefficients of variation. With few exceptions, the coefficients of variation (Tables I to VI and Text-figs. 8 and 9) for consecutive determinations of both calcium and inorganic phosphorus are smaller than those for all observations on a given group of animals (1, 2) and they tend to diminish with successive observations. This, of course, indicates that the variation shown by the animals of a group at a particular time is less than that shown by the group during the course of the experiment and that group results tend to become more and more uniform.

There is, however, an evident relation between the variation of mean values and the magnitude of the coefficients. In general, it may be said that, so long as mean values remain comparatively constant, the coefficient of variation tends to be small, but the occurrence of either a decided increase or decrease in mean values is usually associated with an increase in the coefficient of variation. If the change in mean values is gradual and continuous, the coefficient tends to diminish until the magnitude of the mean values approaches the extreme limits of normal when the coefficient again increases. Further

more, while exceptionally high or low values may have a large coefficient of variation, it will be seen, by reference to Tables I to V, that if a high or low value is maintained, the coefficient is usually small. This is especially true of calcium.

From what has been said, it will be seen that there is no inherent relation between the magnitude of a mean value with respect to normal and its coefficient of variation. The relation found is one that indicates the occurrence of change, and in this sense the coefficient of variation becomes a valuable index of the stability of the animal organism with respect to particular conditions. Thus, a large coefficient or an increase in the coefficient of variation for a given series of calcium determinations indicates not only an uncertainty as to the true mean value, but is clearly indicative of an unstable condition and suggests the probable occurrence of a change, while a small coefficient or a decrease in the coefficient implies stability and uniformity of action.

The most important deduction to be drawn from the analysis of these results with respect to time, is that both the calcium and the inorganic phosphorus in the blood of normal rabbits are subject to progressive and periodic variations which effect not only the absolute but the relative amounts, or the equilibrium between the two substances which, within the limits of normal, is probably of more importance than absolute amounts. In like manner, periodic variations are more important in the present connection, than progressive variations as they provide a better basis for a study of any relation that may exist between changes in the chemical composition of the blood and susceptibility to disease.

The results reported agree with observations originally made by Hess and Lundagen (3) on calcium and inorganic phosphorus in the blood of infants and young children and with the results obtained by Grant and Gates (4) for calcium in the blood of normal rabbits, in so far as the occurrence of variations in one or the other of these substances is concerned. There are, however, certain differences. The variations found by Hess and Lundagen were confined largely to inorganic phosphorus, and no significant variations were found in adults. Moreover, there was a steady reduction in phosphorus from December to March

which was followed by a rise during May and June producing what was termed by them a seasonal tide.

In the experiments of Grant and Gates, the results for inorganic phosphorus were not analyzed. The highest values for calcium were obtained in May and November and the lowest in January, but no results were reported for February, June, July, or August.

In the experiments reported above, periodic variations were more clearly defined in the case of calcium than inorganic phosphorus, but the results differ from those of Grant and Gates as to the time of occurrence of maximum and minimum values. The results for inorganic phosphorus are more difficult to interpret, but it seems that for recently acquired stocks, the lowest value was obtained in January and that the increase began in February. There was, however, little or no further increase during March and April, while the highest value was obtained in June. Among animals living in the laboratory, no period of maximum or minimum values can be fixed for reasons which have been stated above. In one case (Group II) a clearly defined minimum occurred in March and this was succeeded by a definite rise; in other cases, the lowest values occurred in May and during the 1st week of June. And these were succeeded by a slight rise.

Differences such as those cited above may be of no particular importance. At any rate, there is no reason to assume that the course of events in man and animal should agree in all respects, or that variations in the calcium and inorganic phosphorus content of the blood of animals should follow a perfectly uniform course with respect to time. On the contrary, from what is known concerning analogous variations in organic constitution (5) and susceptibility to disease (6), it is to be expected that both the time of occurrence and the extent of any change in the chemical composition of the blood would vary from year to year. Moreover, it has been found that qualitative and quantitative differences will occur even among groups of animals that are under observation at the same time, so that one must reckon with variations in the response of animals, as well as with variations in the action of causative factors.

The point to be emphasized is that systematic or orderly variations in blood calcium and inorganic phosphorus do occur; in some instances,

it may be the calcium; in others, the phosphorus that is affected, or both substances may be affected in varying degrees. In the final analysis, however, it is the effect of these changes on the calcium-phosphorus equilibrium which concerns us most, and while various expressions of this relation may show greater or less change than either calcium or phosphorus, the relations do vary in much the same manner as the absolute amounts of the two substances.

Periodic variations in blood calcium and inorganic phosphorus have been referred to as seasonal variations. Hess and Lundagen attributed the increase and decrease of inorganic phosphorus in the blood of infants to changes in the available amount of ultra-violet radiation but they recognized the possibility that other factors might contribute to the production of these conditions. There is abundant evidence to show that ultra-violet rays exercise a profound influence on the inorganic phosphorus content of the blood of both man and animals, and that certain phases of calcium metabolism are likewise affected but analogous effects can be produced by other means (dietary).

The results obtained in these experiments could be explained in part as light effects. This is particularly true of the changes that occurred in inorganic phosphorus. On the assumption that the inorganic phosphorus in the blood is determined to a large extent by ultra-violet rays of short wave-length, one could account for the variations observed among animals living out of doors and for the progressive decrease that occurred when they were brought into the laboratory and thus deprived of this type of radiation. Among animals living in the laboratory, it would be difficult, however, to account for periodic increases in inorganic phosphorus or even variations in the rate of decrease as an effect of short wave-length ultra-violet radiation, and an explanation of the results obtained for calcium would be even more difficult.

Increasing age and the inactivity incident to cage life might also account for a part of the progressive changes in both calcium and inorganic phosphorus, but not for periodic variations. Moreover, as the rate of decrease in phosphorus, with respect to time, was decidedly irregular, it is not likely that age and cage life *per se* were decisive factors in the production of these changes.

Progressive changes in the chemical composition of the blood might be accounted for in various ways, but under the conditions of these experiments, no satisfactory explanation can be offered for the periodic variations. It is evident that the occurrence of these changes is referable to some condition of life which varies roughly with the seasons and is capable of exerting its influence on animals living indoors as well as out of doors, but not necessarily to an equal degree or in precisely the same manner. It is highly probable that ultra-violet rays, in a positive or negative way, played an important rôle in the production of the results reported, but it is practically certain that this was not the only factor concerned. Stated in a general way, it may be said that, while a number of factors probably contributed to the production of the variations observed, the indications are that the light environment, including visible as well as ultra-violet rays, played an important rôle.

SUMMARY.

Determinations of calcium and inorganic phosphorus were made on the blood of 5 groups of normal animals over periods of 4 to 8 months. The material included animals from recently acquired stocks as well as animals that had been living under laboratory conditions for long periods of time. The results were analyzed with especial reference to the occurrence of periodic and progressive variations in the absolute amounts of calcium and inorganic phosphorus and the relations between the two substances.

It was found that, among animals living in the laboratory, both calcium and inorganic phosphorus, as well as all expressions of the equilibrium between the two substances, exhibited a definite tendency to a progressive increase or decrease, as the case might be, and that clearly defined periodic variations occurred in all classes of animals. The progressive change was most marked in the case of inorganic phosphorus, the periodic change in the case of calcium, while both conditions were clearly shown by various expressions of the relation between calcium and inorganic phosphorus.

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THE RÔLE OF CLASMATOCYTES AND CONNECTIVE TISSUE CELLS IN NON-SPECIFIC LOCAL CUTANEOUS IMMUNITY TO STAPHYLOCOCCUS.

BY S. O. FREEDLANDER, M.D., AND J. A. TOOMEY, M.D.

(From the Divisions of Surgery and Contagious Diseases of Cleveland City Hospital, and the Departments of Surgery and Pediatrics of Western Reserve University, Cleveland, Ohio.)

PLATES 29 TO 32.

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In 1923, Besredka (1-4) stated that he could produce a specific generalized immunity in rabbits and guinea pigs to the subcutaneous or intracutaneous injection of staphylococcus and streptococcus by the injection of specific broth filtrates, or, by the local application to the skin of specific broth dressings.

The immunization was best produced in two ways: (1) by the intracutaneous injections over a small area of either (a) a sterile specific broth filtrate (the organism was grown 10-14 days in broth, filtered and the filtrate reinoculated with the same organism and grown again for 14 days and again filtered) or (b) of a vaccine made by heating a 24 hour broth culture of the specific organism at 60° for 30 minutes; (2) by the application for 24 hours over a limited skin area of compresses moistened by either the sterile specific broth filtrate or the heated killed broth vaccine.

The reaction to a subcutaneous injection of 1-2 cc. of a 24 hour broth culture in the control animal was a large sloughing ulcer, whereas in the protected animal, there was only a small localized abscess. Others (5) repeated these experiments with similar results. Gratia (6), however, obtained the same results with broth. By intracutaneous injection, Mallory and Marble (7) produced a local cutaneous immunity to staphylococcus in rabbits with sterile broth as well as with the broth filtrate. Miller (8) protected guinea pigs against staphylococcus with specific broth filtrates, vaccines, horse serum, broth and concentrated meat broth, all of these substances being injected intracutaneously or applied as compresses. The immunity was well localized, spreading but slightly beyond the area treated and in no sense considered specific. Since Besredka's work, clinicians have treated infections of various kinds with specific broth filtrates or vaccines in broth. Citron and Picard (9, 10) review the work done by others and claim to have had good

results themselves in the treatment of erysipelas, furunculosis, carbuncles and osteomyelitis. They used polyvalent broth filtrates and take for granted that the action is specific.

The aim of the following experiments was (1) to investigate the effect of local broth compresses, (2) to determine how constant this protection was and (3) to endeavor to throw some light on the mechanism of the protection.

Method.

Staphylococcus aureus (our laboratory number 35), isolated from a case of human osteomyelitis, was grown on agar for 48 hours and suspended in saline just before injections. In a few experiments, the staphylococcus was grown in broth. In any one experiment, the suspension from all the tubes, obtained by adding 2 cc. of normal saline to each tube, was pooled and a sample of this standardized roughly by centrifugation in a Hopkins tube for 5 minutes at 2000 revolutions per minute (odometer). It was found that a dose of .015-.06 cc. of bacteria suspended in 3 cc. of saline when injected subcutaneously in the abdominal wall or shoulder of a guinea pig produced, in over 66.1 per cent of the control animals, the characteristic sloughing lesion to be described later. The same dose was used in all the animals of any one experiment, and in all instances, the suspension was shaken immediately before the animals were injected. The control and "compressed" animals were injected alternately.

The organism was grown in broth for 10-14 days, filtered through a Berkefeld filter, the filtrate reinoculated for 14 days and refiltered to produce Besredka's filtrate.

Compresses were made of 6-8 layers of gauze, large enough to cover the abdomen and held in place by adhesive strips for 48 hours previous to the injection. They were kept moistened by frequent applications of the various substances used.

Compresses were the only method of protection used. All the experiments were on guinea pigs weighing from 210-250 gm. The broth used in poulticing was the usual laboratory beef extract broth (3 gm. Liebig's Beef Extract, 5 gm. sodium chloride, 10 gm. peptone (Fairchild's), 1000 cc. distilled water—boiled 1 hour, filtered, titrated to pH 7.6 and sterilized).

The Lesion.

The injection of the standard dose of bacteria subcutaneously in the abdominal wall caused death in 23 of the 121 control animals. 80 of the remaining 98 showed a diffuse cystic swelling covering the entire abdomen which appeared during the first 24 hours. The ani-

mals were sluggish and did not take nourishment. Redness, induration and pain were conspicuously absent at this time. During the next 48-72 hours, the edges of this swelling became firm, indurated, red, painful and warm to the touch. The skin in the center became discolored and frequently broke open exuding a serosanguineous material. In 5-7 days, the whole central area sloughed, leaving an ulcer, 2-3 cm. or more in diameter, with raised indurated edges and a granulating base. Healing took place in 3-4 weeks. This lesion we called 3 plus (+++).

Compresses of broth or filtrate applied to the shaved abdominal wall for 48 hours made the skin wrinkled, thick and slightly boggy. 24 hours after the usual subcutaneous injection of *Staphylococcus aureus*, there was a diffuse induration, red and painful, quite in contrast to the soft cystic swelling in the control animal. There was little general reaction as evidenced by activity or feeding. In most of the animals, a small localized swelling, $\frac{1}{4}$ - $1\frac{1}{2}$ cm. in diameter, appeared by the 3rd or 4th day. This eventually discharged thick creamy pus or healed by resolution. This lesion is called 1 plus (+). In many of these cases, the lesion was a minute nodule containing a drop of pus.

In some of the animals the abscess formed was much larger and ran longitudinally up and down the midline, with thick indurated edges. After several days, this broke down discharging creamy pus. This lesion is called 2 plus (++).

To recapitulate, the large ulcerative lesion is called 3 plus (+++), the long abscess 2 plus (++) and the nodule or localized abscess 1 plus (+).

RESULTS.

Experiments show that ordinary meat broth is just as effective in altering the control reaction as the specific filtrate (Experiments 16a and 70). Furthermore, dry compresses and compresses moistened with water or saline give some protection, but it is not as complete as that given by broth (Experiments 19 and 64a).

The protection obtained was localized, for when animals were compressed with broth on the abdomen for 48 hours and then injected elsewhere in the shoulder region as in our experiments, the reaction

was as great in these animals as it was in the controls that had no compresses (Experiments 11a, 16b, 64b).

To determine if the protection afforded by broth was constant, a number of experiments were done (Nos. 18, 30, 34, 35, 38, 39, 41, 58, 66, 71). Of 70 controls, 9 died, while 51 of the remaining 61 showed large ulcerative lesions (3 plus, +++). Of 66 treated with broth prior to injection, 4 died; while of the remaining 62, only 2 showed a 3 plus (+++) lesion; 3, a 2 plus (++) lesion; and 57 had small localized abscesses. If all experiments with broth are totaled, of 121 controls, 23 (19.0 per cent) died and 80 (66.1 per cent) had 3 plus (+++) ulcerations. Among the 116 broth "compressed" animals, there were 7 deaths (6.0 per cent), while 98 (84.4 per cent) had localized abscesses.

To determine how long immunity lasted, animals were injected at various intervals after removal of the broth compresses. Although the experiments were not conclusive, there was evidence that the protection lasted more than 24 hours and less than 7 days.

It is evident from these experiments that guinea pigs can be protected in a fairly constant manner against the subcutaneous injection of *Staphylococcus aureus* by the local application of broth compresses.

In order to observe the histological changes accompanying the foregoing phenomena, control animals and animals which had been treated with broth compresses for 48 hours were killed at various intervals after bacterial injection and sections were taken of the abdominal wall. Animals previously injected with trypan blue intraperitoneally (once daily with 3 cc. of a 1 per cent solution for 4 days) were similarly treated. In all, 218 guinea pigs were used for histological study.

Sections included (1) normal abdominal wall, (2) abdominal wall after 48 hours of broth compressing, (3) the abdominal wall from control and "compressed" animals at varying intervals after bacterial injection (6-9 hours, 18-30 hours, 72 hours, 96 hours, 120 hours, 6 days and 10 days), (4) duplicate sections from animals previously injected with trypan blue.

The animals were killed by injecting formalin into the heart and the specimens obtained were washed and fixed in the usual manner before being imbedded in paraffin. All sections were stained with hematoxylin and eosin. In addition from the specimens vitally stained with trypan blue, one section was mounted unstained and one was faintly counterstained with carmine.

Normal Skin.

The normal skin of the guinea pig presented no unusual features. The epidermis was approximately 2-4 cells in thickness, the corium had the usual compact papillary and lower reticular layers. Of most interest for our purpose was the narrow reticular zone of subcutis contained between the hair follicles and the striated muscle layer. In this layer, were distinguished the two chief types of cells found in the connective tissue—the elongated connective tissue type with a large elliptical, compact, dark staining nucleus and a small amount of cytoplasm and the clasmatocyte with an irregular, indefinite outline, a large eccentric nucleus and cytoplasm filled with vacuoles of various sizes. The latter cells, the clasmatocytes, were often clustered at nodal points in the reticulum. They took up trypan blue in the vacuoles. A few dye-containing cells were scattered through the corium, but the majority were in the subcutis.

After Broth Compress for 48 Hours.

After an animal had been “compressed” with broth for 48 hours, the demonstrable changes were: (1) thickening of the epidermis, (2) edema of the subcutis, (3) a striking proliferation of clasmatocytes and connective tissue cells in the subcutis and (4) a moderate exudation of small mononuclear and polymorphonuclear leucocytes.

The epidermis was definitely thickened, comprising 4-8 cell layers. There was an edema of both corium and subcutis, most striking in the latter, for while in the normal animal it was only about one-fifth of the width of the corium, now it had become of equal breadth. All through the skin there was an increased number of cells most marked in the subcutis. While there was a moderate number of polymorphonuclears and small mononuclear leucocytes, there was, especially in the subcutis, a marked increase in the number and size of the clasmatocytes and the elongated connective tissue cells. The tissue macrophages were often of enormous size with a very definite outline that was more rounded in this than in the resting stage. A large vesicular nucleus rather poor in chromatin was pushed eccentrically by the rich vacuolization of the cytoplasm. Occasionally they contained two nuclei. There was no striking evidence of mitosis in any section. Trypan blue was taken up in large amounts by the tissue macrophages and segregated and concentrated in the vacuoles. Although the cells were most dense in the subcutis, a fairly even distribution through the corium might be taken as evidence of motility. The clasmatocytes were enmeshed in a whorl of connective tissue cells

which were also distinctly increased in number and size. There were many small mononuclear cells of various sizes and shapes, grading from the smallest with a compact, eccentric, often bean-shaped nucleus and clear cytoplasm to larger cells with a more vesiculated nucleus, whose cytoplasm often contained a few vacuoles. The blood vessels of the lower corium were dilated and contained polymorphonuclear leucocytes, mononuclear cells and red cells. The endothelium was swollen and showed slight signs of proliferation. Occasionally around the capillary was found a cluster of small mononuclear cells.

The reaction of the skin to a broth compress took place largely in the subcutis as an edema accompanied by a striking increase of clasmato-cytes and connective tissue cells. There was also a distinct thickening of the epidermis.

6-9 Hours after Bacterial Injection.

(a) *Controls.*—The control animals presented a tremendous edema of all layers of the skin, extending down through the connective tissue muscle sheaths. The epidermis was thinned, the papillæ flattened and the hair follicles compressed. Polymorphonuclear leucocytes were scattered throughout the corium and subcutis in large numbers. They were filled with bacteria, but showed definite signs of degeneration, *i.e.*, haziness of cell outlines, pycnosis, and fragmentation of the nuclei. There were relatively few mononuclear cells, most of which were small. Clasmatocytes were scarcely to be found, a fact which was confirmed in the trypan blue specimens which showed that very few cells ingested the dye. Connective tissue cells were not increased in number. Scattered through the edema, were many extravasated red blood cells. At this stage there were very few extracellular bacteria.

(b) *Broth-Protected.*—In the animals treated with broth compresses, the histological picture was quite different. The epidermis was still more thickened, the papillæ were prominent and the hair follicles well preserved. Edema was present throughout, although not as marked as in the control animals. The cellular infiltration, however, was much denser, particularly in the subcutis. Polymorphonuclear leucocytes predominated. They were filled with bacteria, but showed no signs of degeneration as evidenced by staining reactions. Small mononuclear cells of the types previously described were present in

very large numbers. There were many clasmatoocytes scattered through and at the periphery of the exudate. The number of fibroblasts was increased so definitely as to suggest beginning organization even at this early stage in the lesion. In the trypan blue sections, the clasmatoocytes at the periphery of the lesion took up the dye in large amounts, while in the most central part of the exudate, these macrophages were often filled with bacteria and phagocytosed blood cells, so that the dye previously ingested was scattered through the cell. The blood vessels were dilated and congested and the swollen endothelium showed signs of proliferation. The differences between the control and the broth animals were found chiefly in the subcutis. In the broth-protected animal there was (1) a proliferation of clasmatoocytes, (2) a proliferation of fibroblasts with beginning organization and (3) the preservation of the integrity of the polymorphonuclear leucocytes. There was also a marked thickening of the epidermis.

21-30 Hours after Bacterial Injection.

(a) *Controls.*—In the control animals, the epidermis showed signs of compression, often being lifted from the corium. The whole corium stained poorly and was disintegrating. The edema, still marked throughout the subcutis, had not increased, but the polymorphonuclears present showed further signs of degeneration. There was no attempt at organization. In the trypan blue sections, practically no cells took the dye. The epidermis, however, was stained diffusely, a sign of necrosis. There were few extracellular bacteria.

(b) *Broth-Protected.*—In the broth-prepared animal, the epidermis was still further increased in thickness. The edema was decreased, but the cell exudation was richer in the subcutis. A definite zone of proliferating fibroblasts was beginning to circumscribe the lesion. Polymorphonuclear leucocytes, increased in number, still retained their definite cell outlines and nuclear delineations. Among the fibroblasts were large numbers of clasmatoocytes with richly vacuolated cytoplasm, often enormous in size and occasionally having two nuclei. Trypan blue was found abundantly in these cells. Many clasmatoocytes had phagocytosed bacteria, polymorphonuclear leucocytes and red blood corpuscles and showed only scattered dye granules. The blood

vessels were full, the endothelium swollen and proliferating and frequently endothelial lining cells jutted into the lumen of the vessel.

Rapidly advancing organization due to proliferating fibroblasts, together with marked activity of the clasmatocytes which were beginning to act as scavengers were the most marked changes at the end of 21-30 hours.

48-72 Hours after Bacterial Injection.

(a) *Controls.*—The control animal showed a marked degeneration of epidermis and corium. In the subcutis, the edema had decreased with no increase in cellular infiltration. The polymorphonuclears showed advanced signs of disintegration and bacteria, which previously were practically all intracellular, were now found in large numbers outside of the cells. Frequently large bacterial masses were seen, suggesting *in vivo* multiplication. Beginning signs of organization were seen in the lower part of the subcutis. In this zone, when the specimen had been stained with trypan blue, occasional cells took up the dye.

(b) *Broth-Protected.*—The broth animals showed a little greater thickening of the epidermis with broad deep papillæ. In the corium, there was a moderate cell increase, while in the subcutis there was a definitely walled off abscess. The zone of organization composed of fibroblasts and macrophages was thick. A large number of the cells in the peripheral portion of the exudate was enclosed in macrophages which were often engorged with phagocyted polymorphonuclears and debris. This was further shown by the fact that in the trypan blue specimens, few cells, except those at the extreme periphery, ingested the dye in large amounts, while it was present in many cells as scattered granules. The number of smaller mononuclear cells was decreased, while the number of large mononuclears was increased.

4-10 Days after Bacterial Injection.

(a) *Controls.*—In the control animals, the corium and epidermis had sloughed, leaving an ulcer with an organizing base just above the partially or wholly disintegrated muscle layer. In this zone, many cells now showed some avidity for trypan blue granules.

(b) *Broth-Prepared*.—In the broth-prepared animal, the abscess had definitely localized itself in the subcutis. Eventually, it either came to the surface and was evacuated or the clasmatoocytes took up the cellular exudate and ingested it, while the fibroblasts proliferated through the lesion and organized it. At the end of 10 days, the clasmatoocytes had largely digested their burden and the cytoplasm again showed marked vacuolization, so that the trypan blue was distributed in large amounts in the vacuoles.

To summarize, broth causes a marked stimulation of the cells of the subcutis, the clasmatoocytes and the connective tissue cells, which seemed to protect the animal against the overwhelming effects of the bacteria. The macrophages phagocyted bacteria, thus relieving the burden imposed upon the polymorphonuclears so that they in turn were not destroyed by the infection. In addition, the clasmatoocytes later phagocyted the polymorphonuclear leucocytes before they could disintegrate and free the bacteria. Concomitant with this was the rapid proliferation of fibroblasts walling off the lesion. The origin of the smaller mononuclear cells is difficult to determine. However, it was evident that they may develop into larger phagocytic cells and thus contribute largely to the number of these cells seen in the subacute stage of the lesion.

COMMENT.

Inasmuch as there is no question of specificity involved in these experiments, it remains to correlate the histological changes, *i.e.*, the increase of clasmatoocytes, fibroblasts and round cells, with the marked reduction in mortality and the definite alteration of the inflammatory reaction.

While it is generally recognized that the tissue macrophages play a part in the subacute stages of inflammation, it has not been emphasized until recently, that they can offer an effective barrier to bacterial infection.

Metchnikoff (11) and his pupils considered the large mononuclear leucocytes of the blood as the typical macrophages and thought that the large cells in the connective tissue were of lesser importance. In experimental streptococcus infection (12), they believed that the phagocytes of the connective tissue had nothing to do with the disposal of bacteria except in so far as they engulfed polymorphonuclear leucocytes which contained them.

In experimental inflammation of the subcutaneous tissue, both aseptic and bacterial, Maximow (13, 14) definitely separated the clasmatocytes from the fibroblasts by their marked difference in motility and phagocytic activity. Macrophages which were present in large numbers at an early stage in the inflammation, especially the aseptic lesion, he thought came from three sources, (1) through proliferation of fixed tissue macrophages, *i.e.*, clasmatocytes, (2) through the development from the polyblasts or small mononuclears present in the tissue and (3) by the development from lymphocytes and mononuclear cells coming in from the blood stream.

Tschaschin (15) confirmed these findings in experimental peritonitis. Many others (for full reference, see Gay (16)) have observed the early appearance of large phagocytic cells in peritoneal inflammation. Following the work of Evans and Scott (17) who established definite vital staining reactions for clasmatocytes as opposed to the connective tissue cell, it was determined that these phagocytic cells in the peritoneal exudate were of connective tissue origin (Cunningham (18)).

While Rous and Jones (19) and Smith, Willis and Lewis (20) showed that in tissue culture, clasmatocytes have marked phagocytic activity, it remained for Gay and Morrison (21) and Gay and Clark (22) to definitely link these cells with immunity. They found that substances such as infusion broth or diluted egg white, locally injected, produced a marked increase in the number of clasmatocytes in the pleural cavity of rabbits. Such an animal was thus protected against many times the fatal infective dose of streptococcus when injected intrapleurally. In the normal animal, the streptococci increased until the death of the animal (5-7 days), while in the broth-prepared animal, the cultures from the pleural cavity were sterile in from 3-4 hours. Substances such as aleuronat which produced largely an increase in polymorphonuclear leucocytes, gave no such protection. After bacterial injection, rabbits actively or passively immunized against streptococci showed a much earlier mobilization of clasmatocytes in the pleural cavity than control animals.

We cannot discuss the much disputed relationship between clasmatocytes, large mononuclears of the blood and endothelial cells. However, we believe that the large phagocytic cells seen after broth compresses are proliferated largely from the clasmatocytes of the connective tissue. Morphologically, they are similar, their staining reaction the same and their rapid development into large cells with richly vacuolated cytoplasm is different from the gradual change of the small mononuclear cells into phagocytic cells even under the added stimulus of bacteria. There is no doubt that these latter cells contribute to the number of macrophages later seen in the lesion. Furthermore, broth compresses cause a concomitant proliferation of the elongated connective tissue cells. This is evidence of the stimulation of the connective tissue cell as a whole.

The rapid increase in the number of fibroblasts plays an important part in the protective reaction. Their function would seem to complement the activity of the clasmotocytes. The latter phagocyte the bacteria early, while the fibroblasts organizing around the focus hinder the spread of noxious material before it can be taken up by the phagocytic cells.

Polymorphonuclear leucocytes and small mononuclear cells certainly play a part later in the reaction. But these cell types, however, are too few in number directly after broth compressing and before bacterial injection for one to imagine that they are the effective agents in increasing the resistance.

After bacterial injection, the early disintegration of the polymorphonuclears in the control animal is of importance. This allows a proliferation of the organisms and a recrudescence of their activity before the clasmotocytes are present in large enough number to assist by ingesting the degenerating leucocytes. We infer that the presence of a large number of clasmotocytes previous to injection in the broth animal not only diminishes the virulence of the bacterial attack by the phagocytosis of the organisms, but by ingesting the leucocytes containing staphylococci, they prevent a recurrence of bacterial activity.

SUMMARY AND CONCLUSION.

1. Plain broth is just as effective as specific broth filtrate if used as a skin compress for the protection of guinea pigs against a subcutaneous injection of *Staphylococcus aureus*.

2. Plain broth compresses applied for 48 hours previous to bacterial injection sometimes prevent the death of the animal and practically always alter the inflammatory reactions.

3. This protection is not specific and is localized to the area "compressed."

4. The protection lasts at least 24 hours after removal of the compress.

5. Broth compresses applied to the abdominal wall of a guinea pig for 48 hours produced definite histological changes, especially in the subcutis, *i.e.*, edema, proliferation of clasmotocytes, thickening of the epidermis together with a moderate exudation of polymorphonuclears and small mononuclear cells.

6. The histological response to the subcutaneous injection of staphylococci was different in the control and the broth-prepared animal.

7. In the broth-prepared animal, there was an increase in clasmato-cytes and fibroblasts with a dense exudation of polymorphonuclears, which latter, in the main, did not degenerate. The clasmatocytes phagocyted bacteria early and later engulfed the polymorphonuclears, while the fibroblasts rapidly walled off the lesion. The result was a localized abscess which either came to the surface and ruptured or was absorbed and organized.

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EXPLANATION OF PLATES.

PLATE 29.

All sections described have been stained with hematoxylin and eosin.

FIG. 1. Section through normal abdominal wall. Low power.

FIG. 2. Section through abdominal wall after broth compresses had been applied for 48 hours. Low power.

FIG. 3. Section through abdominal wall of control animal (not "compressed") 48 hours after bacterial injection. Low power.

FIG. 4. Section through abdominal wall of broth "compressed" animal 48 hours after bacterial injection. Low power.

FIG. 5. Section through abdominal wall of control animal (not "compressed") 96 hours after bacterial injection. Low power.

FIG. 6. Section through abdominal wall of broth "compressed" animal 96 hours after bacterial injection. Low power.

FIG. 7. Section through abdominal wall of control animal (not "compressed") 6 days after bacterial injection, showing ulceration. Low power.

FIG. 8. Section through abdominal wall of broth "compressed" animal 6 days after bacterial injection. Low power.

PLATE 30.

FIG. 9. Section taken after broth compresses had been applied for 48 hours. Shows clasmotocytes, fibroblasts and mononuclear cells. Oil \times about 475.

FIG. 10. Control animal (not "compressed") 8 hours after bacterial injection. Shows degeneration of exudate cells. \times about 325.

FIG. 11. Broth "compressed" animal 8 hours after bacterial injection. Shows exudate cells well preserved and a proliferation of fibroblasts. \times about 325.

FIG. 12. Same as Fig. 10. Oil \times about 475.

PLATE 31.

FIG. 13. Same as Fig. 11. Shows clasmotocytes containing bacteria. Oil \times about 475.

FIG. 14. Animal (not "compressed") 21 hours after bacterial injection. Shows marked degeneration of exudate cells. \times about 325.

FIG. 15. Broth "compressed" animal 21 hours after bacterial injection. Shows many clasmotocytes, fibroblasts, small mononuclear cells. Cells of exudate well preserved. \times about 325.

FIG. 16. Same as Fig. 15. Oil \times about 475.

PLATE 32.

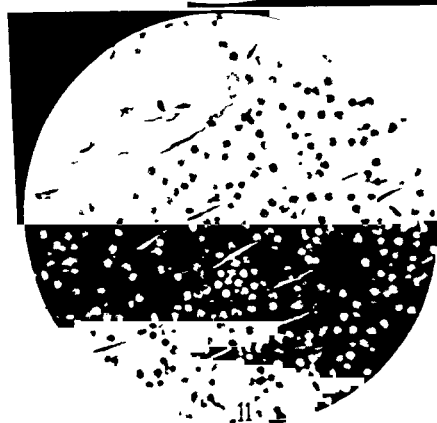
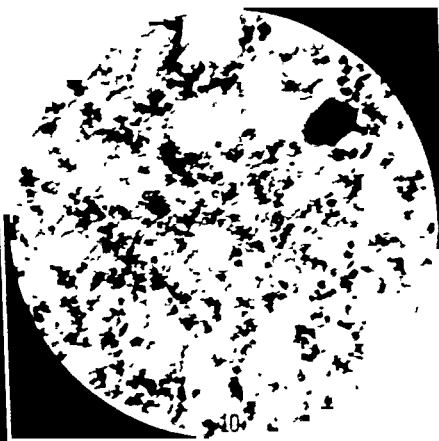
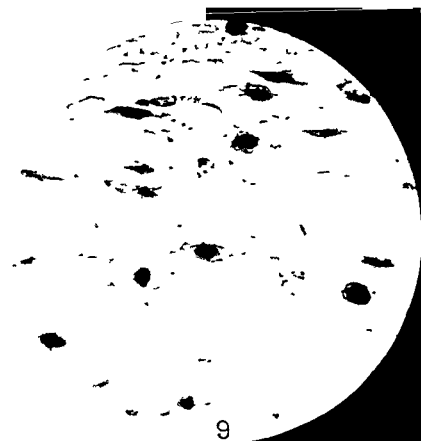
FIG. 17. Control animal (not "compressed") 72 hours after bacterial injection. Shows marked cellular degeneration and extracellular bacterial proliferation. Oil \times about 950.

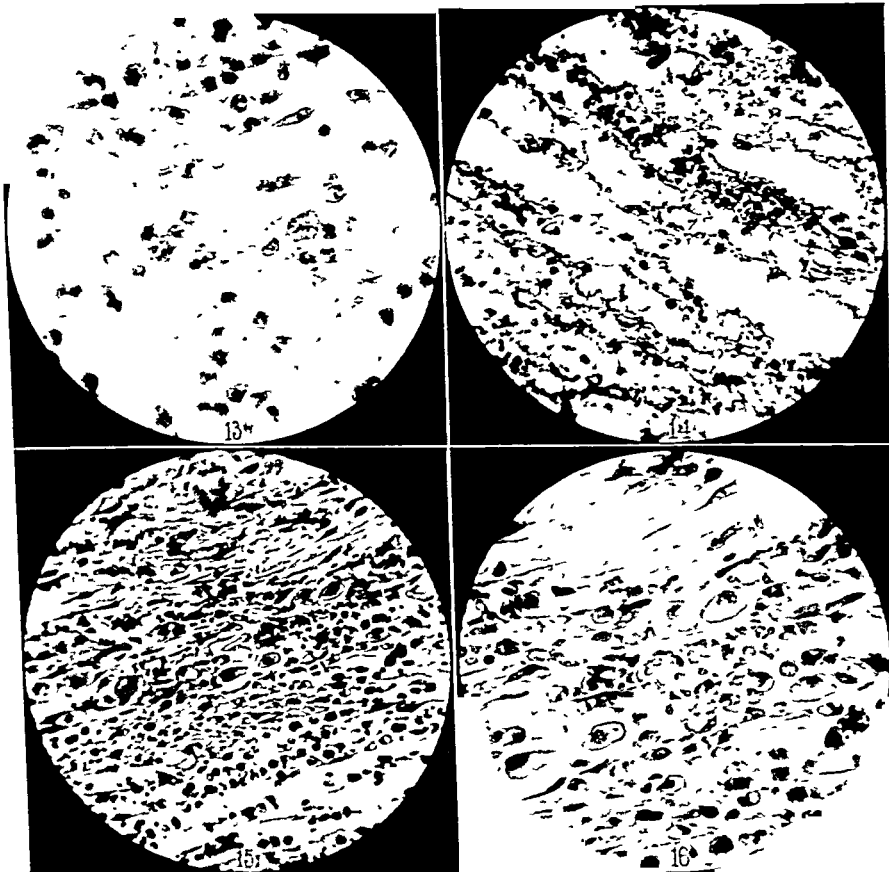
FIG. 18. Broth "compressed" animal 72 hours after bacterial injection. Shows phagocytosis of the cells of the exudate by macrophages. Oil \times about 950.

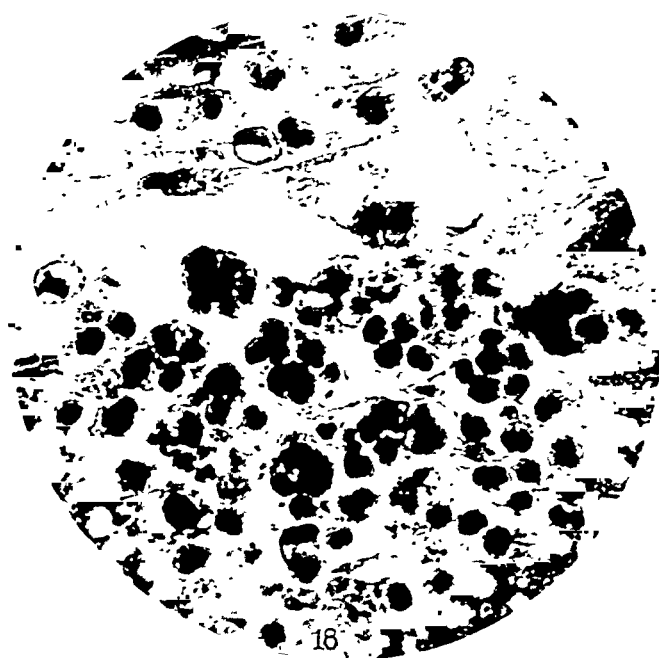
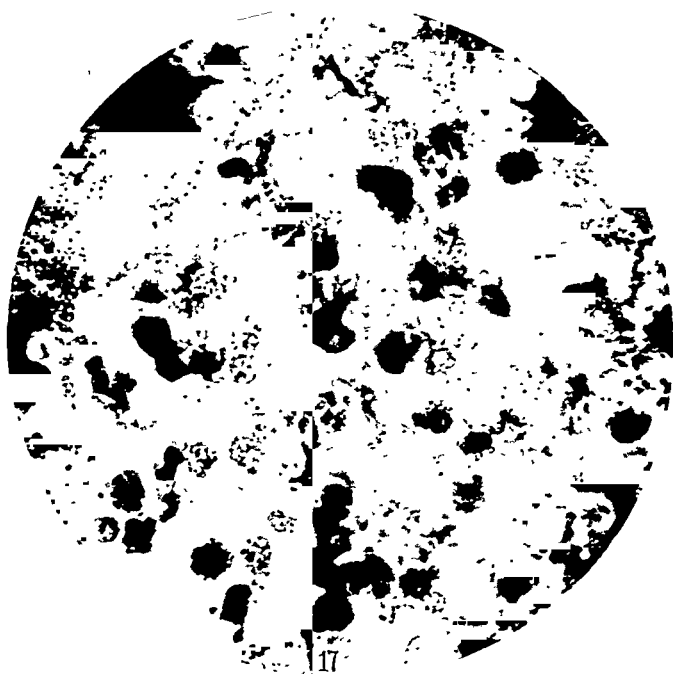


(Freedlander and Toomey. Local cutaneous immunity to staphylococcus.)









(Freedlander and Toomey: Local cutaneous immunity to staphylococcus.)

THE ELECTROPHORESIS OF THE BLOOD PLATELETS OF THE HORSE WITH REFERENCE TO THEIR ORIGIN AND TO THROMBUS FORMATION.

By HAROLD A. ABRAMSON,* M.D.

(From the Kaiser Wilhelm Institute for Physical Chemistry and Electrochemistry, Berlin-Dahlem, Germany.)

(Received for publication, January 23, 1928.)

Little is known of the physicochemical characteristics of blood platelets. There has been much dispute concerning their origin. The study of their cataphoresis affords a means of determining a fairly definite physicochemical constant which defines the make-up of these bodies in specific channels. Such data on the cataphoresis of the blood platelets of the horse and some other incidental observations, form the basis of this communication.

Review of the Literature Concerning Origin of Platelets.

The following review merely indicates the variance of opinion. It is incomplete.

In 1906 Wright (1) maintained that the cytoplasm of megacaryocytes was parent to that of blood platelets. Bunting (2) and Ogata (3) confirmed Wright's work. Brown (1913) (4), however, showed that hyperplastic endothelial cells in the marrow, and mononuclear and transitional cells in the marrow, spleen and blood, could also give rise to blood platelets.

Menne (5) made a specific immune serum from leucocytes and platelets and concluded from his studies that the structures of the leucocyte and platelet vary. Perroncito (6) (1920) believed that platelets arise from the red and white cells of the circulating blood. Schilling (7) (1921) came to the conclusion that the nuclei of red cells play a significant rôle in platelet formation. Erede (8) (1921) rejected the megacaryocyte origin. Rosenthal and Falkenheim (9) (1922) performed rather careful and well controlled experiments. They found that an erythrocytic immune serum was highly agglutinative for red cells but had a comparatively

* Work completed during the tenure of a Medical Fellowship of the National Research Council.

negligible influence on platelets. Platelet antisera also failed to agglutinate red cells. They concluded that, from the point of view of receptor structure, platelets and red cells present significant differences, while with the cells of the leucopoietic system the platelets possess a common receptor system indicating a common origin for the platelets. Marchesini (10) (1923) maintained that platelets arise from degenerated red blood cells which are phagocyted by megacaryocytes and then transformed by these cells into the form found in the blood. A rather complex hypothesis has been offered by Demel (11). This theory seems to hold for the origin of these cells (platelets) by a precipitation process direct from the blood plasma. The process is to be governed by megacaryocytes in the presence of physiological necessity. Further evidence in support of Wright's view was given by Katsunuma (12) (1925). Petri (13) in the same year denied the validity of Wright's views. Stahl, Horstman and Hilsnitz (14) by means of an iodine fixation method showed that certain granules were specific for platelets and megacaryocytes, again supporting Wright.

Method.

A modification of the Northrop (15) cataphoresis cell described elsewhere by Freundlich and Abramson (16) was used.

The plasma was oxalated by the addition of 8.5 cc. of a saturated solution of K oxalate per liter of horse blood. The platelets remain well preserved in the ice box for at least 48 hours.

The velocity of the platelets and of the polymorphonuclear leucocytes was determined as follows: The study of red cell migration at different levels in the cell permits the estimation of V_w , the velocity of the water in the midregions. The velocity of another particle in the midregions is then expressed by the equation

$$V_o - V_w = V \quad (1)$$

where V_o is the observed velocity of the particles and V is the absolute velocity. The reader is referred to previous communications for further data on method and related phenomena of cataphoresis (16, 19).

The Migration of Single Platelets.

The data of Table I are from six different horses. The mean velocity for the platelets is $.45 \mu$ per sec. per volt per cm. Polymorphonuclear leucocytes migrate with the same speed, within the limits of experimental error. In fact, simple observation confirms the measurements. Practically no difference in speed is observed. It may be recalled that in plasma lymphocytes migrate 15 to 30 per cent, and red cells about 90 per cent faster than leucocytes (19). The

same relationship holds therefore for blood platelets. Considering the difficulties of the method, the high conductivity of the medium and the low electrokinetic potential, with the exception of Plasma 2, the agreement in the five other specimens is excellent. It is surprising that blood platelets which are supposed to be so fragile retain for so long the same surface characteristics as far as the electrokinetic po-

TABLE I.

The Cataphoresis of Platelets in Plasma.

The speed of polymorphonuclear leucocytes is given in the last column. Although red cells and small lymphocytes have different velocities, note that platelets and polymorphonuclear leucocytes have the same velocity (six horses).

Plasma	Age	Platelets		Polymorphonuclear leucocytes V
		V_0	V	
	<i>hrs.</i>	μ per sec. per volt per cm.	μ per sec. per volt per cm.	μ per sec. per volt per cm.
1	30	.71	.41	.46
2	6	.82	.59	.57
	30	.76	.55	.60
3	6	.65	.46	.52
4	6	.57	.40	.43
5	6	.67	.51	.53
6	6	.68	.46	.54
Mean excluding No. 2			.45	.49
ζ potential (millivolts)			12	13
(26.5 $\times \mu$ per sec. per volt per cm.)				

tential is determined by this surface. One is almost led to believe that their surface is determined by the presence of the plasma proteins rather than by an inherent composition.

The Migration of Aggregates of Platelets.

According to classical conceptions (17) the ζ potential of agglutinated blood platelets should be lower than that of single cells. In

specimens 30 hours old clumps of from 5 to about 20 platelets have been studied. There is no appreciable difference between the cataphoretic velocity of these aggregates and that of single cells. It is possible that some change in the ζ potential takes place incidental to the withdrawal of the blood. Just how far the aggregates observed represent a mechanism of slow coagulation by the particles below the critical potential must be reserved for future discussion.¹ The formation of aggregates without change in electrokinetic potential has been reported previously by Freundlich and Abramson for red cells (16).

Another outstanding feature of platelets is that they are able to stick to the glass walls of the cataphoresis cell with a remarkable tenacity. (That platelets are "sticky" has, of course, been noted hitherto.) This force is so great that a stream of water sucked through the cell does not remove them. The same adhesive quality has been discussed previously for leucocytes (19, 20). On the other hand, it is curious that neither red cells nor lymphocytes under the same conditions are possessed of similar properties. This fits in remarkably well with the behavior of all four types of cells in the presence of injury to tissue or capillary wall.²

DISCUSSION.

It would seem from the preceding data that the surface of polymorphonuclear leucocytes and that of blood platelets are similar. The electrokinetic potential is the same for both in a highly complex medium. This is all the more striking because of the fact that lymphocytes and red cells have a cataphoretic velocity which is unmistakably greater. Offhand one is inclined to believe that the platelets and leucocytes have a common leucopoietic origin. This would fit in quite nicely with the theory of Wright as follows: The blood platelets arise from the megacaryocytes which have in their

¹ Polymorphonuclear leucocytes and even quartz particles form aggregates whose cataphoretic velocity is the same as that of single particles with the same suspension. This "isopotential" agglutination will be discussed further in a future communication.

² This point is discussed in detail in an article by the author to be published (Abramson, H. A., in Alexander, J., *Colloid chemistry*, New York, ii).

turn been derived from myeloblastic cells (21); and the transition from myeloblast to leucocyte is, as far as concerns surface change, probably not a complex one. Still, one should accept this rather convincing evidence with a certain amount of hesitancy as it has been found in further experiments that quartz particles migrate with the same velocity in serum as leucocytes.³ It has been also found that such quartz particles are influenced by slight traces of proteins (e.g. 10^{-7} gelatin solution lowers the ζ potential of quartz appreciably (18, 22)). Now, whether the white cells and blood platelets act like an inert particle, surrounding their naked protoplasm or cell membrane with a sheath of the protein in the medium, or whether they have acquired during their development the surface giving them their charge, is a question which is intimately bound with studies of surface adsorption and cataphoresis of these blood units. Experiments on the point have been started. At any rate, one can say that platelets and leucocytes have similar surfaces—certainly slightly different from lymphocytes and very different from red cells. And one may assume with a fairly reasonable degree of certainty that the unchanged relationship through the development of both types is strongly suggestive of a common origin.

The stickiness of the blood platelets has been noted. It must be remarked in concluding that the so called glass surface of the cataphoresis cell is really covered by a more or less complete layer of protein in the presence of even small protein concentrations (18). The platelets are really adherent to a protein film. The magnitude of this adhesive force has in general been described. It has been mentioned that aggregates are formed without changing the ζ potential. With this in mind, one may look upon thrombosis from the following point of view. Incidental to injury of the vessel wall, the adhesive force possessed by platelets is sufficient to permit them to remain attached to the wall in spite of the flow of blood rushing by. No change in electrokinetic potential is needed to establish a state of aggregation. Aggregation can probably occur in plasma without any measurable change in the ζ potential. The piling up of blood platelets can easily be explained

³ The data and connected theory will be given in Abramson, H. A., *J. Gen. Physiol.*, 1928, xi, in press.

by the stickiness of the cells themselves, produced by adsorbed or inherent protein films. The same mechanical, simple conception may be applied to the subsequent addition of leucocytes to the thrombus and the attachment of fibrin strands.

SUMMARY AND CONCLUSIONS.

1. The cataphoretic velocity of blood platelets (horse) in plasma has been found to be between $.40$ and $.51 \mu$ per sec. per volt per cm. The mean velocity obtained from five horses is $.45 \mu$ per sec. per volt per cm.

2. The cataphoretic velocity of polymorphonuclear leucocytes in similar specimens is practically identical with that of the platelets. This is noteworthy because of the fact that lymphocytes and red cells have different speeds.

3. With spontaneous agglutination of platelets, white cells and red cells, there is no change in the cataphoretic velocity incidental to aggregation.

4. The possible surface composition of platelets and white cells is briefly discussed.

5. The bearing of these findings on the origin of blood platelets and the mechanism of thrombus formation is demonstrated.

I am indebted to Professor H. Freundlich for his criticisms and advice.

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THE MODE OF SPREAD OF A FRIEDLÄNDER BACILLUS-LIKE RESPIRATORY INFECTION OF MICE.

By LESLIE T. WEBSTER, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

PLATE 33.

(Received for publication, January 27, 1928.)

During observations of spontaneous *B. enteritidis* infection among special populations of mice (1, *a*), we noted the sudden appearance of a second disease which killed a great many of the animals. It arose first as an epidemic in late August, 1925, continuing in waves during the autumn and winter, and finally, in the spring, disappearing completely. A second similar outbreak occurred in August, 1926. The dead mice showed at autopsy bilateral pneumonia, and yielded in cultures from blood and lung an organism resembling Friedländer's bacillus.

The disease, in its sudden onset, high morbidity and mortality rates, its recurring outbreaks decreasing in severity, and its eventual disappearance, suggested somewhat human plague of the middle ages and the recent epidemic of influenza. For this reason, it was chosen for study, and its manifestations analyzed according to the experimental methods which we have employed in the investigation of mouse typhoid (1, *b*). The present paper describes the clinical course, pathology, and bacteriology of the disease, and records certain special studies of reactions between the bacteria and their host.

The literature on Friedländer bacillus-like infections, save for that relating to pneumonias of human beings, is scant and indefinite. Human case reports of Friedländer bacillus lesions of various organs, especially lesions situated near the upper respiratory tract, are numerous, however. Abel and Hallwachs (2) give references in which are described the recovery of this class of organisms from the atmosphere, earth, dust, water, and slime. Animal strains were reported by Pfeiffer in 1889 (guinea pigs (3)); Klein in 1889 and 1891 (guinea pigs and mice (4)); Weaver in 1897-99 (guinea pigs (5)); Skschivan in 1900, 1903 (rats (6)); Perkins in 1901 (guinea pigs (7)); Sachs in 1902 (rats (8)); Schilling in 1902 (rats

(9)); Toyama in 1903 (rats (10)); Aujeszky in 1904 (rats (11)), and Nylander in 1906 (rats (12)). Nylander refers also to communications on the subject by Fricke, Wilde, Fasching, and Gaffky. In reading these reports, however, one receives the impression that the organisms described form a rather heterogeneous group of capsulated bacteria, the effects on animals of which, were in many instances, quite different from that associated with typical strains of Friedländer's bacillus.

Since 1912, little has been written on Friedländer bacillus infection of animals, except for two articles by Holman (1916 (13)) and Branch (1927 (14)), in which the spontaneous disease in guinea pigs was described.

The general features of the disease in mice, as observed by us, are as follows:

Clinical Course.—The incubation period is about 48 hours. When a few carriers are placed in contact with groups of 100 or more mice, a certain number dies within 5 days, while 50-70 per cent are dead within 2 weeks. An occasional animal with signs and symptoms of pneumonia recovers, while a small number seems to resist infection altogether. Similarly, when a culture of the bacilli is introduced artificially into the nares of mice, a few animals die on the 2nd day, 70 per cent by the end of the 1st week, and a small number survives as nasal carriers or appears entirely refractory.

Pathology.—In mice dying of this infection, the autopsy shows subserous, petechial hemorrhages, such as occur in other septicemic infections, and extensive inflammation of the respiratory tract. The lungs are inflated, uniformly red and moist, and the serosa is covered with a seropurulent exudate. On section the lung tissue is red and wet, contains little air, and the exuded fluid is viscid and "stringy."

The microscopic lesions vary in extent and degree. In most instances there is dilatation of the interstitial capillaries, with or without accompanying interstitial hemorrhage and edema. Many alveoli contain serum and, occasionally, red blood cells. Beneath the pleura the congestion and edema are especially prominent. In many cases there is a fibrinous cellular exudate over the surface endothelium. The more advanced specimens show perivascular, interstitial, and intraalveolar accumulations of mononuclear and a few polymorphonuclear cells. Blood vessel walls are infiltrated and generally surrounded by cells; bronchi and tubules appear normal.

When groups of mice are given small doses of the organisms (200-600 intranasally), the pulmonary lesions described above are reproduced, and the differences observed are found to be related directly to the duration of life of the animals inoculated. For instance, mice dying 48 hours after the instillation show perivascular accumulations of large numbers of bacilli, perivascular round cell infiltration, and interstitial congestion; while those surviving 4 to 5 days show fewer bacteria, diffuse interstitial congestion and edema, and accumulations of leucocytes. After a longer period the alveoli are filled with leucocytes.

The pneumonic condition is in general similar to that described by Branch in spontaneous guinea pig infections (14) and by Stillman and Branch in experimental Friedländer infection in mice (15). The nature of the lesions studied by us suggests that the inoculated bacilli reach the lungs by way of the blood stream rather than by the bronchi (1, d).

Bacteriology.—The Friedländer bacillus-like organisms may be cultured from the nasal passages, lungs, and blood. Except in cases of severe septicemia, they are not found in the intestinal tract. Under dark-field illumination they appear as large, non-motile, homogeneous, blunt rods. They are Gram-negative; the capsules stain vividly,

TABLE I.

Cross-Agglutination Tests Made by Dr. Julianelle with His A, B, C, and X Friedländer Strains.

Strain	Friedländer type sera: dilution 1:5					Mouse Strain 1. Serum 1:5
	Type A	Type B	Type C	Group X I	Group X II	
Mouse Strain 1.....	—	—	—	—	—	+++
“ “ 2.....	—	—	—	—	—	+++
“ “ 3.....	—	—	—	—	—	+++
“ “ 4.....	—	—	—	—	—	+++
“ “ 5.....	—	—	—	—	—	+++
Friedländer Bacillus “A”.....	+++	—	—	—	—	—
“ “ “B”.....	—	+++	—	—	—	—
“ “ “C”.....	—	—	+++	—	—	—
Group X I.....	—	—	—	+++	—	—
“ X II.....	—	—	—	—	+++	—

and Wright's blood stain exhibits a large enveloping material surrounding a deeper staining substance. Dextrose, maltose, xylose, salicin, and mannite are fermented in 24 hours; saccharose in 3 to 4 days, and lactose in 7 to 12 days. Milk is acidified; no indol is formed; nitrates are reduced to nitrites.

The serologic reactions of the strains obtained by us are practically identical. Sera obtained from rabbits by injecting dead and living cultures agglutinated all strains to a maximum titer of 1:640. No antigenic relationship was established with other known strains of Friedländer's bacillus.

A series of cross-agglutinations and thread tests with five of our mouse strains and with Friedländer's bacilli, Type A, B, C, and X, was made for us by Dr. Julianelle (16). Table I gives the results. The five type sera of Friedländer's bacillus failed to agglutinate any of the mouse strains, and the mouse strain serum agglutinated none of Julianelle's five type strains of Friedländer's bacillus.

Colonies of the bacteria growing on agar plates from direct mouse autopsy culture are large, moist, and viscid, "stringing" easily on the platinum loop (Figs. 1 and 2). Bacilli from young, single cell colonies show no mucous material. At a later period, the entire colony, save for the peripheral zone of activity dividing bacteria, is composed of the enlarged cells, which in mass present a mucoid appearance.

Growth requirements of these organisms are relatively simple. No enriching substances, carbohydrates, serum, "X", or vitamine factors need be added to plain infusion agar or broth, pH 7.0-7.4. However, considerable sensitivity to temperature changes exists.

Experiment 1 compares the growth of cultures at 37° and 25°C.

Two series of tubes containing 5 cc. of plain infusion broth of pH 7.4 were inoculated with different dilutions, 10^{-1} to 10^{-9} , of a 48 hour broth culture of Mouse Strain 3. One series was incubated at 37°; the other left at room temperature, 23°. Growth in the two series was compared at 24 and 48 hours. 3 days later the cultures were examined by dark-field illumination, by staining methods, and by replating. 6 days after inoculation, the numbers of viable bacteria in each tube of the series were counted by the plating method.

The first four tubes of the 37° series showed good growth at 24 hours; the remaining five, 10^{-5} to 10^{-9} inclusive, were sterile. The entire series grown at 22° were turbid at 24 hours. The original culture used for inoculation contained about 800,000,000 organisms. Hence, as the findings show, an inoculation of 100,000 organisms was required to produce growth at 37°, whereas the smallest number, less than 10, grew at 22°. No further growth occurred at 48 hours. At this time, examination of cultures by dark-field and staining methods showed autolysis, great swelling, and reduction in numbers of bacilli in the tubes of the 37° series; the 22° cultures appeared normal. Counts at 6 days showed less than 100 bacilli per tube in the first four dilutions of the 37° series; the rest were sterile. Plates of tubes of the 22° series averaged about 170,000,000 organisms per cc. per tube.

The tests were repeated with other strains with essentially the same results. Hence, the optimal growth temperature is considerably less than 37°.

The next experiment was planned to ascertain the duration of life of these organisms in fluid culture.

Experiment 2.—Four flasks with 300 cc. of broth in each were inoculated with a 48 hour culture of Strain 1. Two received about 12,000 organisms and two about 10; actual counts were made immediately. One flask of each dilution was then incubated at 37°; the two others were left at room temperature, 22°. Counts were made of the numbers of bacteria in each flask at 3, 6, 25, and 52 hours, and at 10 months. The results are shown in Table II.

TABLE II.

Growth of Small Numbers of Friedländer-Like Bacilli at 37° and 23°C.

Time after inoculation	No. bacteria per cc.			
	Flask 1: inoculation 10 ⁻² 22°	Flask 2: inoculation 10 ⁻² 37°	Flask 3: inoculation 10 ⁻⁴ 22°	Flask 4: inoculation 10 ⁻⁴ 37°
<i>hrs.</i>				
0	12,000	15,000	10	10
3	48,000	9,000	0	0
6	70,000	3,000	30	0
25	180,000,000	0	18,000,000	0
52	325,000,000	0	280,000,000	0
<i>mos.</i>				
10	6,000,000*	0	5,000,000*	

* Rough variants 100 per cent. No mucoid forms.

Both incubated (37°) flasks were sterile at 24 hours and remained so. The two flasks standing at 22°, room temperature, showed good growth at 25 hours and viable organisms 10 months later. At this time, however, the mucoid colonies were replaced entirely by the rough variant colony type. Hence these cultures are found to be relatively simple in their requirements and able to survive for long periods of time at temperatures of about 20–24°C. Higher temperatures, however, prove harmful.

Colony type variation occurs readily with the Friedländer group of bacilli. Hadley and Julianelle have summarized the reports of early investigators. Hadley (17) found that rough colonies of Friedländer bacillus arise when cultures are grown for some days in broth or agar. Branch witnessed the same phenomenon in the cultures from guinea pigs (14). Julianelle noted transformations in his A, B, and

C type strains grown in antiserum (16). Our mouse cultures lose the mucoid colony appearance when grown in antiserum, and when kept 2 or more weeks in broth or agar. The variant colonies are non-mucoid, quite small, opaque, with regular margins and granular surfaces (Figs. 1, 2, 3).

The variant mouse strain colonies are not found under natural conditions. During the past 2½ years, we have cultured the nasal passages of more than 2000 mice and autopsied at least as many more, and obtained only mucoid colony forms. We conclude, therefore, that the variant types do not occur in recognizable numbers during either the interepidemic or epidemic phase of the native mouse disease.

Single cell cultures from the variant colonies are relatively stable in spite of various manipulations *in vitro* and *in vivo*, a reverse transformation from variant to the original mucoid form was not accomplished.

Bacilli from the variant colonies are smaller than those from the mucoid colonies, and lack the enveloping material; otherwise the two resemble each other. The short rods are Gram-negative and non-motile, fermenting the characteristic sugars, acidifying milk, and reducing nitrates to nitrites. They agglutinate in antimucoid sera to more than double titer. They are not flocculated by sera from Julianelle's Type A, B, and C strains.

Microbic Virulence and Host Susceptibility.

We have determined the effects of different numbers of bacilli on the amount and severity of the experimental infection, and have made titrations of the virulence of the same and different bacterial strains, over a period of more than 2 years.

Technique.—The general principles governing the technical procedures of these titrations have been described elsewhere (1, b). Briefly, we consider it essential to reproduce the natural conditions of infection as nearly as possible. The dosage, virulence, and host susceptibility tests are planned in such a way as to remove as far as possible disturbing variables. Thus, under given conditions, relatively constant results are secured. The circumstances most nearly reproducing the native infection are adopted as a standard. This

standard is then used for comparison for titrations of dosage, virulence, and host factors.

Cultures are grown in a uniform medium and treated alike throughout. Dosage is estimated in numbers of bacilli administered. The bacilli, suspended in a drop of salt solution, are introduced through a small glass pipette into the nasal passages of each mouse. The spasmodic inhalations of the animal aid the taking in of the fluid. Mice from the inbred Rockefeller Institute stock, raised under uniform environmental conditions, about 12 weeks of age, weighing 18-20 gm., and not previously exposed to the infection are used (1, c). The inoculated mice are placed in separate cages.

Standard Curve.—At the outset, the precision of the titration technique was determined by performing a number of titrations in duplicate and observing the amount of variation in the end results.

Technique.—Strain 1, obtained on October 25, 1926, from a mouse in one of the special populations, was used throughout. For each titration, a subculture in broth was made from the stock slant and grown for 48 hours at 23°. A dose of 200-600 bacteria per mouse was found to represent natural conditions most satisfactorily. Hence each animal was given intranasally, by glass capillary tube, 1 drop of a 1:10,000 0.85 per cent salt dilution. This volume contained 200-600 organisms, as checked each time by dilution plates. The mice were chosen according to the above specifications. After inoculation, each animal was numbered and placed in a separate jar. The entire number was then divided arbitrarily into groups of 20, 25, 50, or 100, according to the amount used, and the mortality in each group was compared. Dead mice were autopsied and cultured. The results of these tests are summarized in Table III.

On November 16, 1926, a titration was made with 50 mice, divided into two groups of twenty-five. Each group showed close agreement in amount and rate of death. 84 per cent were dead in each on the 18th day; none died thereafter (Table III). On November 22, a similar test was made, resulting in a final mortality on the 42nd day of 86 per cent, with a variation between groups of 6 per cent. A third titration on December 2 with three different doses and six groups of twenty mice each showed total mortalities of 92.5 per cent with a group variation of 2.5 per cent (1250 bacteria per mouse), 90 per cent total and group mortality (380 bacteria per mouse), and 55 per cent, with a variation between groups of 10 per cent (58 bacteria per mouse). On December 16, a fourth test with two groups of twenty and a dose of 195 bacilli resulted in the death of 70 per cent total and 5 per cent group variation mortality. A fifth titration on December 20, with two groups of twenty-five and a dose of 960 bacteria per mouse, showed a final mortality of 90 per cent with a 2 per cent variation of group mortality. On January 5, 1927, two groups of 50 each received 15,000 organisms per mouse, and two groups of 50 a dose of 115 bacilli. The mortalities at 42 days were 86 per

cent with a 4 per cent and 57 per cent with a 9 per cent final difference in group mortality.

The results of duplicate titrations, of which the above are a part, showed that the experimental technical error was insignificant; hence tests carried out as described above proved sufficiently accurate.¹ Furthermore, the mortality rate per day was found to be so uniform that average figures were taken to represent numerically the reaction of the Rockefeller Institute strain of mice to a dose of 200-800 bacteria of Strain 1. These total mortality percentages, when plotted against time in days, formed a frequency curve similar to those obtained in mouse typhoid studies (1, *b*) (Text-fig. 1). Hence, measurement of variations in dosage, virulence, and host susceptibility were compared

¹Dr. John W. Gowen analyzed the data contained in Table III and commented upon it as follows: "Mathematically analyzed, using mortality rates and weighing them equally, the variation within the groups is quite insignificant in contrast to the variation between the groups made at different times, indicating that the technique has been satisfactory, thus:

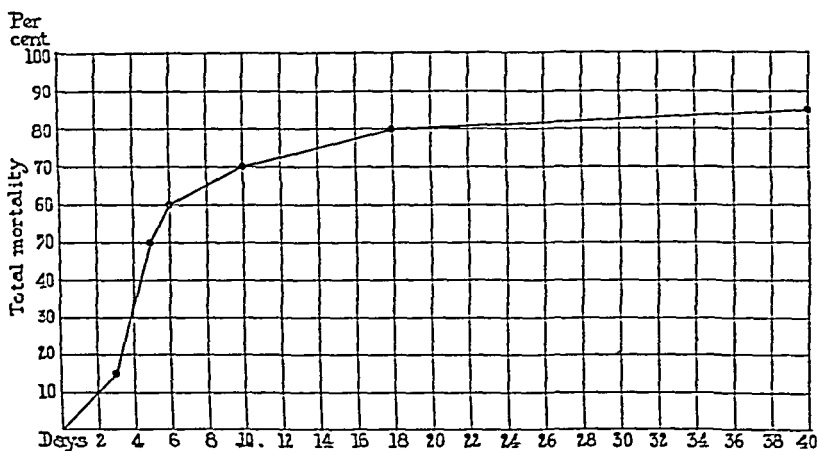
Variance	Degrees of freedom	Mean square
Between groups	9	375.1
Within groups	10	58.7

$Z = .927$ where for $P = .05$ $Z = .56$

"Two possible causes of the significant variation between groups are obvious in the data. The first is variation in dosage, as indicated by the plate counts, and the second is seasonal variation, although the controlled environment of the mice and cultures make such an interpretation doubtful. The data are not well suited to attempts to separate these possibilities since the number of repeated titrations at different dates is not sufficient. However, if the material be drawn as a graph with death rate as one coordinate and dosage of bacteria as the other, it is noticed that for dosages from 58-400 there is a rapid almost linear rise in death rate from 55-390. At 400 bacteria the death rate becomes essentially the same to dosages up to 15,000. This suggests dosage as the important cause of the variation between groups. Approaching the problem slightly differently, there are two dates on which groups of mice were given different dosages, December 2, 1926, and January 5, 1927. Analyzing this data for influence of date of inoculation, the mean variance between dates is found to be 70 and within dates 433. Clearly the date of inoculation played an insignificant part in the death rate. As the cause of variation within the dates is the dosage, we may attribute to it the major rôle in causing the variation in the death rates within these data with influence of season or variation in technique as but minor and insignificant causes of variation."

either with these average figures or with those obtained from a control group tested simultaneously.

Dosage.—Observations of the spontaneous disease among the mice of the special populations indicated that contact with a very small number of the bacilli is fatal to most individuals. For example, surviving carriers in the mouse populations were found to harbor in their nasal passages but very few of the Friedländer bacilli, while mice from which fifty or more colonies were cultured invariably died within a few days. These findings were confirmed by actual titrations which showed



TEXT-FIG. 1.

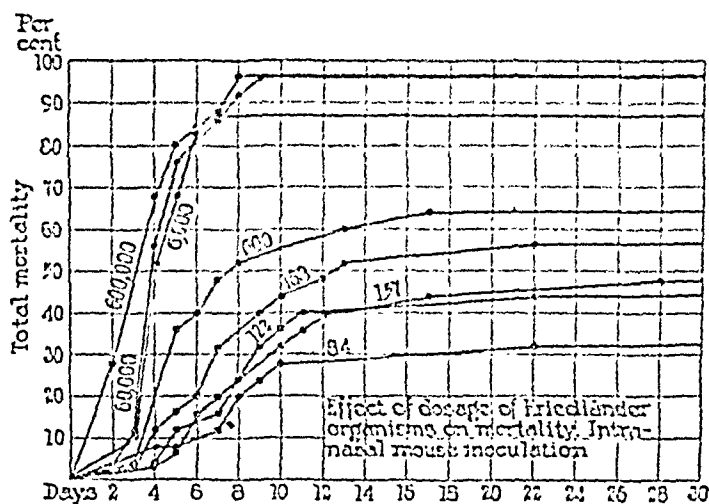
that while a small number of mice survived even a maximal dose, as few as 500 bacilli per mouse killed about 80 per cent within a week. Tests were made to determine this relationship more accurately.

Experiment 3.—Strain 1 was transferred from the stock slant to meat infusion broth, pH 7.4, and left at room temperature (23°) for 48 hours. Dilutions were then made in 0.85 per cent NaCl, counted by the plate method, and given intranasally to mice by means of a coarse capillary pipette. Eight dilutions were used; each was given to twenty-five mice. After inoculation, the animals were placed in separate jars. Mortality records were kept and autopsies done on all dead animals.

The duration of life of the individuals of each group is plotted in Text-fig. 2. The mortality rates of groups receiving 6,000, 60,000, and

600,000 bacilli were quite similar. No deaths occurred after the 9th day; one animal of the 600,000 and 60,000, and three of the 6000 group survived 30 days. In the remaining groups death rate and total mortality were related closely to dosage. 61 per cent of the group given 600 bacteria succumbed, 56 per cent of those given 168, 44 per cent of those given 122, and 32 per cent of those receiving 84.

These tests show that small doses (less than 100 bacilli), given to a group of twenty-five mice, are fatal to certain individuals; that doses ranging from this to over 1000 lead to a progressively increasing death rate and total mortality in groups of similar size; and that doses larger



TEXT-FIG. 2.

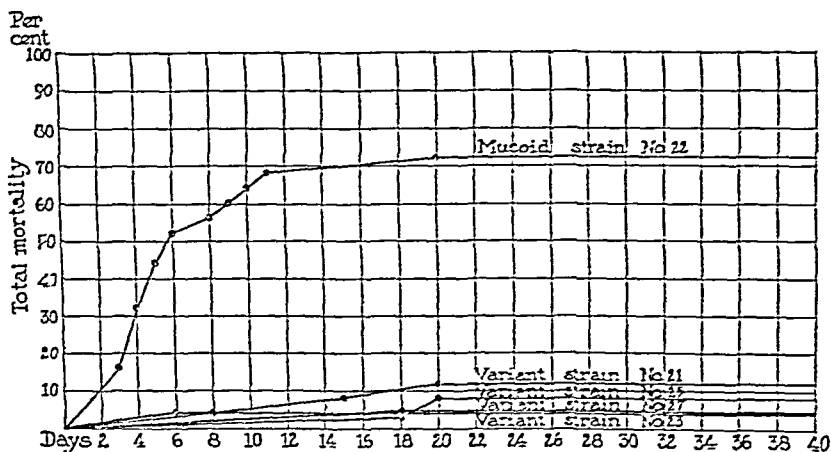
than 6000 organisms do not increase the number of deaths beyond a certain point. Regardless of the size of the dose, some individuals survive.

Virulence.—The virulence of the bacilli has been determined by tests similar to those described above. The standard dose of about 600 organisms was instilled intranasally. Sufficient animals were used to run each titration in duplicate. Strains of unknown pathogenicity were compared directly with known cultures, or indirectly by means of the standard curve described above (Text-fig. 1).

Repeated titrations of single strains over a period of 12 months showed no significant changes in virulence. The results of the tests

on Strain 1 are given in Table III. This culture has been kept on agar at 4°C. and transferred every 4 weeks. It was obtained from a mouse of a known susceptible race (1, c), 2 days before the onset of a severe spontaneous epidemic. The degree of virulence of the type-pure culture was found to remain relatively constant. Three other strains kept type-pure and tested in the same way showed insignificant fluctuations in virulence.

Rough variants obtained from old broth or agar cultures proved far less virulent than the parent mucoid type. The following test illustrates this difference.



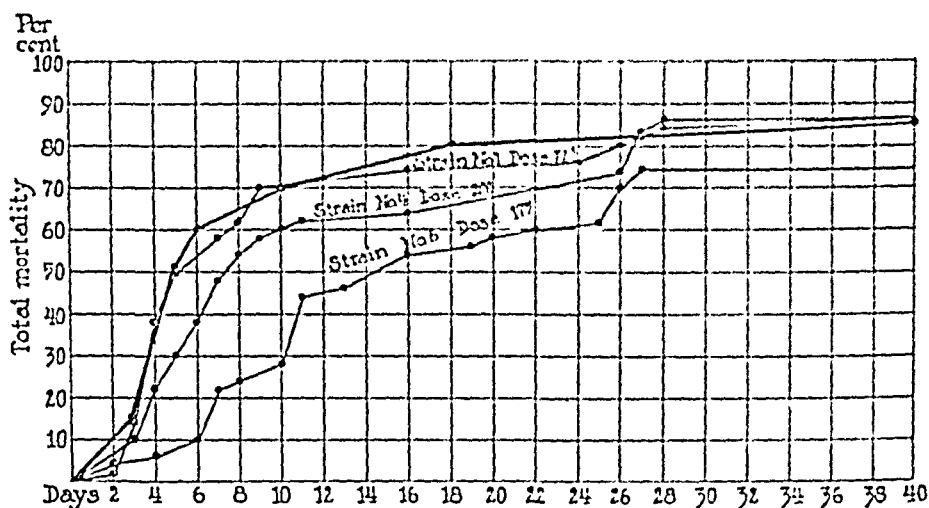
TEXT-FIG. 3.

A mucoid type strain, No. 22, obtained from a mouse of one of the special populations, September, 1925, its rough variant, No. 23, and three other rough variants from mucoid type strains isolated at about the same time were grown 48 hours in broth at 22°C., diluted 1:10,000, and administered intranasally to mice. Each culture was given to twenty-five individuals. The mortality record is plotted in Text-fig. 3.

The mucoid culture, No. 22, showed high virulence characteristic of the type, while its rough variant, No. 23, together with the three other rough strains, Nos. 21, 25, and 27, killed only very few of the animals—4.4, 12, 8, and 4.4 per cent respectively.

A number of titrations were made of cultures taken from mice of the

special populations in which the spontaneous infection was prevalent in endemic and epidemic form. The strains were chosen under varying conditions, with a view to determining differences in lethal power coincident with high or low population mortality, with cultivation of the bacilli in the circulating blood of susceptible individuals, and in the nasal passages of animals resisting infection. A chart describing the course of the infection among the special populations, with a record of the days on which these cultures were obtained, will be published later; at present it will suffice to state briefly the amount of disease prevailing at the time each culture was obtained.



TEXT-FIG. 4.

The method of titration of virulence described in a previous paragraph was employed throughout. Dilutions were made to insure a dosage of about 600 bacilli per mouse. Due to slight irregularities of growth, however, the actual counts as determined by plating varied considerably from this number. Such fluctuation in the relatively small doses of these organisms given has been shown to affect death rate considerably (Experiment 3), and it is responsible, we believe, for the irregularities in some of the virulence curves.

The results of the virulence trials with unknown strains have been compared either with the standard control curve for Strain 1, or with a simultaneous control titration with Culture 1. Each group of mice

TABLE IV.

Mortality during Days after Inoculation.

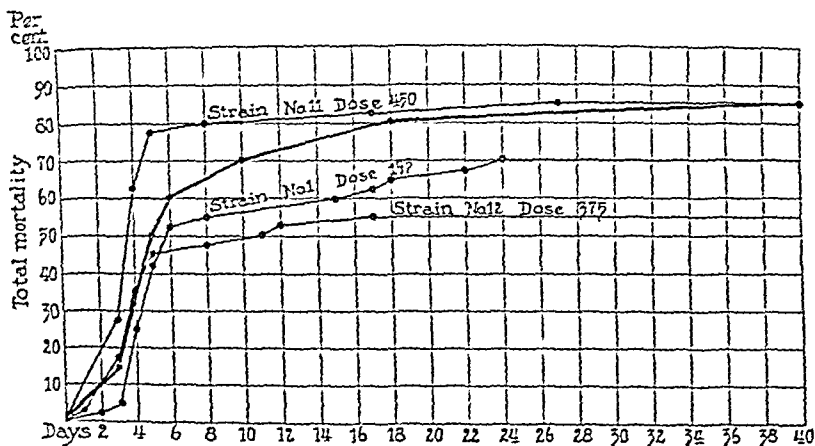
			0-2	2	4	6	8	10	12	14	16	18	20	22	24	26	28-30	Total	Death rate	Average age at death		
Date	Dose	Group																				
11-22-26	720	1- 25			8	3	2	2			2					1	1	19	76	9.95±	1.07	
Strain 1		26- 50		1	10	3	4	2									2	1	23	92	9.35±	1.04
		Total		1	18	6	6	4			2					1	2	2	42	84	9.61±	.75
Strain 4	200	1- 25			8	4	4	1	1		1						2	2	23	92	11.17±	1.16
		26- 50			3	4	4	2									3	4	20	80	14.90±	1.49
		Total			11	8	8	3	1		1						5	6	43	86	12.91±	.95
Strain 6	175	1- 25				1	5		4	1	2		2				3	2	20	80	16.70±	1.12
		26- 50		2	1	1	2	2	4		2				1		2		17	68	13.00±	1.17
		Total		2	1	2	7	2	8	1	4		2	1			5	2	37	74	15.00±	.83
12-16-26	450	1- 20			15	2	1											18	90	5.44±	.17	
Strain 11		21- 40			10	4						1						1	16	80	7.88±	1.08
		Total			25	6	1					1						1	34	85	6.59±	.53
Strain 1	195	1- 20			4	5	1				1				1	1		13	65	9.92±	1.26	
		21- 40		1	5	6					1	2						15	75	8.33±	.90	
		Total		1	9	11	1				2	2			1	1		28	70	9.07±	.76	
Strain 12	375	1- 20		1	7	2			2			1						13	65	7.46±	.83	
		21- 40			6	2	1						1					9	45	5.89±	.31	
		Total		1	13	4	1		2			1						22	55	6.82±	.52	
12-20-26	960	1- 25		1	12	8	1											22	88	5.82±	.19	
Strain 1		26- 50		2	9	9	2	1										23	92	6.22±	.26	
		Total		3	21	17	3	1										45	90	6.02±	.16	
Strain 13	225	1- 25			6	11	4			1								22	88	7.18±	.31	
		26- 50			7	5	6											18	72	6.89±	.27	
		Total			13	16	10			1								40	80	7.05±	.21	
Strain 15	580	1- 25			9	5	1	1	1	1								18	72	7.11±	.47	
		26- 50			11	5		1									1	18	72	7.22±	.87	
		Total			20	10	1	2	1	1							1	36	72	7.17±	.49	
1- 7-27	150	1- 25		1	6	7	2	8										24	96	7.83±	.36	
Strain 17		26- 50			6	11	3	2										22	88	7.09±	.25	
		Total		1	12	18	5	10										46	92	7.48±	.22	

TABLE IV—*Concluded.*

			0-2	2	4	6	8	10	12	14	16	18	20	22	24	26	28-30	Total	Death rate	Average age at death	
Date	Dose	Group																			
1-7-27	187	1- 50			23	13	2	1	2					1				42	84	6.76±	.34
Strain 1		51-100			22	11	1	1	1					1				37	74	6.57±	.36
		Total			45	24	3	2	3					2				79	79	6.67±	.25
	110	1- 25			9	5	1							1	1			17	68	8.06±	.97
Strain 16		26- 50			12	5	1			1								19	76	6.26±	.37
		Total			21	10	2			1				1	1			36	72	7.11±	.51
1-19-27	400	1- 50			14	17	6	3	1					1				42	84	7.43±	.33
Strain 18		51-100			3	8	4	6	4	2				1				28	56	9.93±	.49
		Total			17	25	10	9	5	2				2				70	70	8.43±	.29
	630	1- 50			7	9	7	4	3							1		31	62	8.68±	.47
Strain 19		51-100			5	19	3	4	1	1				1				34	68	8.24±	.40
		Total			12	28	10	8	4	1				1	1			65	65	8.45±	.31
2-28-27	500	1- 25			10	9		1		1								21	84	6.62±	.35
Strain 34		26- 50			12	8												20	80	5.80±	.15
		Total			22	17		1		1								41	82	6.22±	.20
	590	1- 25			9	8	2	1										20	80	6.50±	.25
Strain 19		26- 50			9	7	3					1	1					21	84	7.67±	.62
		Total			18	15	5	1				1	1					41	82	7.10±	.35
	320	1- 25			13	3	3											19	76	5.95±	.23
Strain 33		26- 50			10	5	1	1	1		1							19	76	7.11±	.50
		Total			23	8	4	1	1		1							38	76	6.53±	.28
	540	1- 25			12	3	1	1				1						18	72	6.67±	.54
Strain 32		26- 50			9	5	2	1										17	68	6.41±	.29
		Total			21	8	3	2				1						35	70	6.54±	.31
5- 2-27	420	1- 25			13	8	1											22	88	5.91±	.17
Strain 50		26- 50			1	8	6	3										18	72	6.22±	.26
		Total			14	14	4											40	80	6.05±	.15

receiving a given culture was divided arbitrarily into two subgroups of equal numbers, and the mortality of each compared with that of the total group. Hence a mean daily mortality figure was obtained and the extent of deviation from this mean of each subgroup noted.

Test 1. The Virulence of Freshly Isolated and Stock "Epidemic" Strains.—Strain 1 was obtained from the heart's blood of a susceptible Lathrop mouse, October 15, 1926, 2 days before the outbreak of a severe epidemic; Strain 4 from a similar animal, November 17, 1926, just prior to a similar wave. Strain 6 was isolated at the peak of an epidemic, August, 1925, from an individual of a different, though similar population. Since that time, it has been kept on plain agar at 4°C. On November 22, 1926, each strain was given to 50 mice which were then numbered and placed in separate jars. Two groups of twenty-five mice each were made arbitrarily for comparison of results. The dosage employed was as follows: Strain 1, 720 organisms per mouse; Strain 4, 200 organisms per mouse; Strain 6, 175 organisms per mouse. The results are summarized in Text-fig. 4 and Table IV.

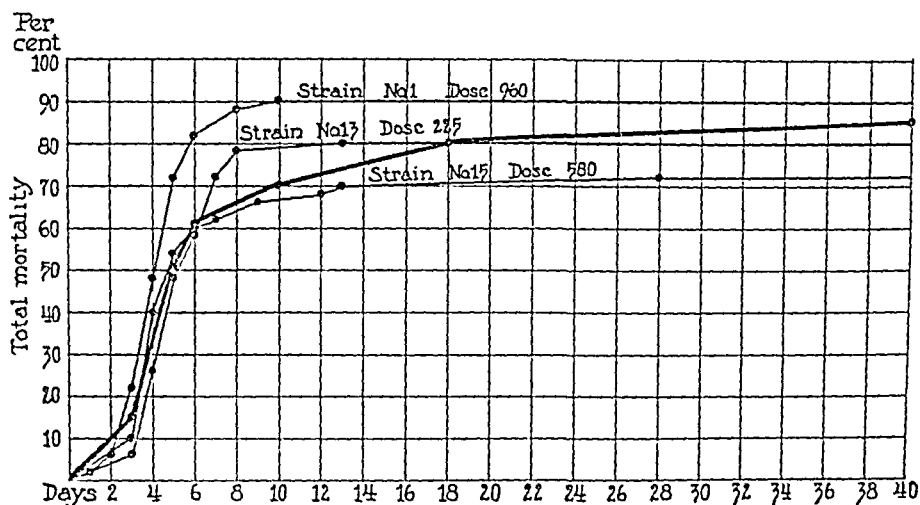


TEXT-FIG. 5.

The mortality of the groups receiving Strains 1 and 4 was similar throughout the period of the experiment. 84 and 86 per cent respectively, were dead on the 40th day. The final deviation from the mean of the subgroups was 8 and 6 per cent, and the average daily deviation 7.4 per cent for mice receiving Strain 1 and 8.6 per cent for those receiving Strain 4. The death rate of the group receiving Strain 6 was lower during the 1st week but deaths continued over a longer period of time. The final mortality was 74 per cent, with a deviation of subgroups from the mean of 6 per cent and an average daily deviation of 1.5 per cent. The mortality curves of the groups receiving Cultures 1 and 4 paralleled closely the standard mortality curve.

The two cultures, therefore, may be considered to be of equal virulence. Culture 6 seemed somewhat less effective, but this discrepancy is believed to be due to differences in actual dosage of bacilli received, rather than to any difference in their degree of virulence.

Test 2. The Virulence of Freshly Isolated "Epidemic" Strains Taken from the Nasal Passages of Healthy Mice.—Three strains were used in this test,—Strain 11 was obtained from the nasal passages of a healthy mouse of the so called "Friedländer" population 1 day prior to the outbreak of a severe epidemic, and Strain 12 from a similar mouse in the same population 1 week before the same outbreak. Strain 1 was again employed as control. Each culture was instilled into forty mice. The dose of Strain 1 proved to be 195 organisms per mouse, of Strain 11, 450, and Strain 12, 375. The results are shown in Text-fig. 5 and Table IV.

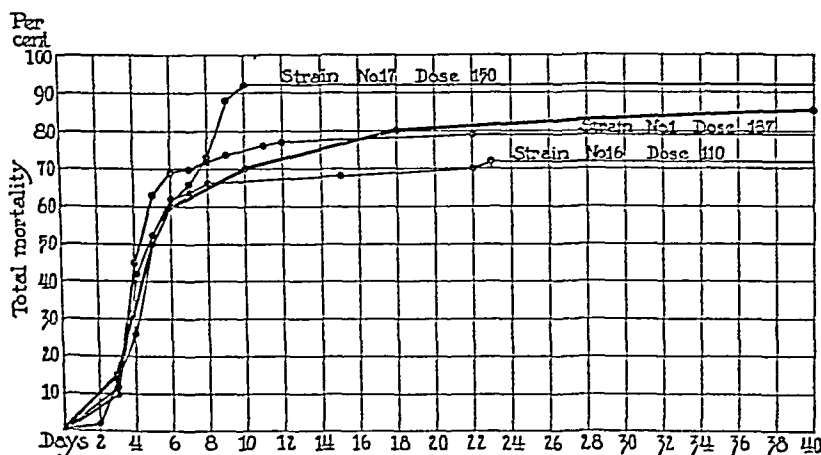


TEXT-FIG. 6.

The final mortality of the group receiving Strain 11 was 85 per cent with a deviation of the subgroups from the mean of 5 per cent and an average daily deviation of 7.4 per cent; that of the group receiving Strain 12 was 55 per cent, with a variation between groups of 10 per cent on the final day, and an average daily deviation of 4.9 per cent, while the final mortality of Strain 1 group was 70 per cent, with a 5 per cent variation and an average daily deviation of 5.6 per cent. The apparent low activity of Strain 12 was due, we believe, to undetermined differences in actual dosage received and not to a difference in virulence.

Test 3. The Virulence of "Epidemic" Strains Taken from the Nasal Passages of Surviving Mice.—Strains 13 and 15 came from individuals of the same population as Strains 11 and 12, and were obtained at the height of the epidemic mentioned above. The mice appeared healthy at the time the cultures were taken and survived the epidemic. Strain 1 was run at the same time as a control. Each culture was given to 50 mice. The dose of Strain 1 proved to be 960 organisms per mouse, of Strain 13, 225, and of Strain 15, 580. The results are summarized in Text-fig. 6 and Table IV.

The mortalities in all three groups were similar and approximated closely the figures of the standard mortality curve. 90 per cent of the Strain 1 group were dead on the 40th day, with the subgroups showing



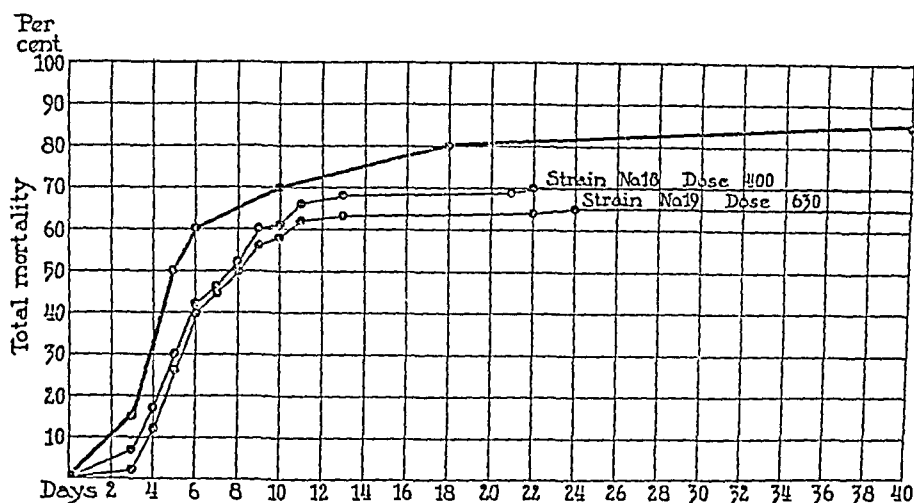
TEXT-FIG. 7.

a final deviation of 2 per cent and an average daily deviation of 2.2 per cent. The mortality of the group receiving Strain 13 was 80 per cent, subgroup variation 8 per cent, and an average daily deviation of 7.2 per cent, and that of the group given the No. 15 culture was 72 per cent, with subgroups identical at the end of the test and showing an average daily deviation of 1.3 per cent. The three cultures tested, therefore, may be considered as equally virulent.

Test 4. The Virulence of "Epidemic" Strains Taken from Heart's Blood of Infected Individuals.—Strain 16 came from a susceptible mouse of one of the special populations designated "G" population, at the height of a severe and long epidemic. Strain 17 came from a mouse of another special population designated

"Friedländer" population, also during the plateau of a severe and long spontaneous epidemic wave. Strain 1 was run simultaneously as a control. One hundred and eighty-seven bacilli of Strain 1 were given to each of 100 mice, 110 of Strain 16 to each of 50 mice, and 150 of Strain 17 to each of 50 mice. The results are shown graphically in Text-fig. 7 and Table IV.

The mortality curves of all three series are similar and follow quite closely the standard control curve. The final mortality of the Strain 1 group was 79 per cent, with a subgroup variation of 5 per cent and an average daily deviation of 4.3 per cent; of the Strain 16 group, 72 per cent, subgroup difference 4 per cent, and average daily deviation 5.2



TEXT-FIG. 8.

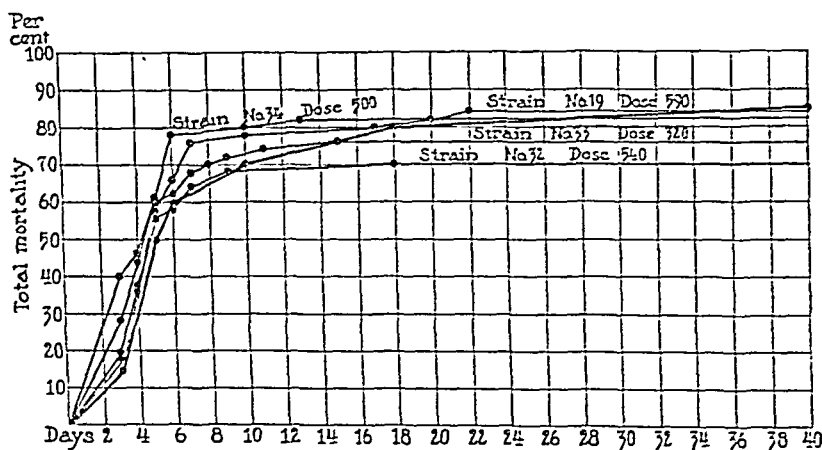
per cent, and of the Strain 17 group 92 per cent, subgroup variation 4 per cent, and average daily deviation 4.2 per cent. The three cultures therefore may be considered to possess equal virulence.

Test 5. The Virulence of "Epidemic" Strains from the Nasal Passages and Circulating Blood.—Strain 18 was obtained from the nasal passages of a healthy mouse of the special "G" population at the height of a severe epidemic. The individual from which this culture was taken survived. Strain 19 came from the blood of a mouse of "No. 6" population, dying at the height of a severe epidemic wave. Each culture was administered to 100 mice in doses of 400 and 630 organisms per mouse respectively. The results are charted in Text-fig. 8 and Table IV.

The mortality of each group was almost identical, and differed from that of the standard control curve by a small margin. The final

mortality of the No. 18 group was 70 per cent, subgroup difference, 14 per cent, average daily deviation 14 per cent; that of the No. 19 group was 65 per cent, with a subgroup variation of 3 per cent, and an average daily deviation of 2.9 per cent. For the instance of the large deviations in subgroups from the mean in the case of the No. 18 group, which is unique, we offer no present explanation. Nevertheless, these cultures may be considered equal to each other and to agree with Strain 1 in virulence.

Test 6. The Virulence of "Epidemic" and "Surviving" Strains.—Strain 19 was described in Test 5. Strains 32 and 33 came from the lungs of two mice of the



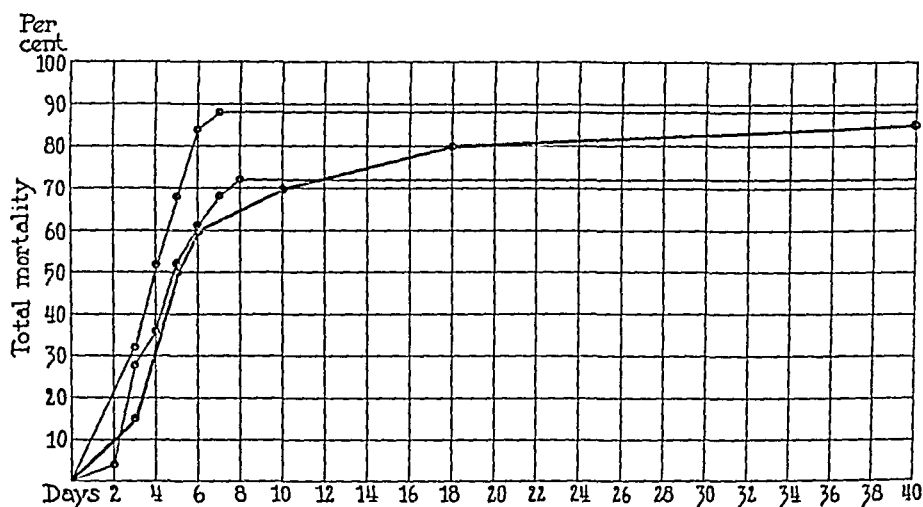
TEXT-FIG. 9.

"No. 6" population dying at the peak of a severe epidemic. Strain 34 came from the nasal passages of an individual of the same population, which had lived throughout the entire epidemic and was surviving and healthy in the interepidemic period at the time the culture was taken. Each strain was administered to 50 mice,—590 organisms per mouse of No. 19, 540 of No. 32, 320 of No. 33, and 500 of No. 34, the results of which are shown in Text-fig. 9 and Table IV.

The mortality curves resemble one another and the standard control curve of Strain 1. 82 per cent of the No. 19 group were dead on the 40th day. The final deviation of the subgroup was 4 per cent, and the average deviation 2.7 per cent per day. The final mortality of the No. 32 group was 70 per cent, with a final subgroup deviation of 2 per

cent and an average daily deviation of 1.5 per cent. That of the No. 33 group was 76 per cent, with no final difference in the subgroups and an average daily deviation of only 1 per cent; that of the No. 34 group was 82 per cent, with a final and average daily deviation of 2 per cent. These strains and Strain 1, then, are considered to be of the same virulence.

Test 7. The Virulence of a Surviving Culture, Strain 35.—This strain was obtained from the only carrier of the "No. 10" population, surviving a severe epidemic wave which had occurred 2 months previously. Within a week after the culture had been obtained from this mouse, it ceased to yield the Friedländer



TEXT-FIG. 10.

bacilli and at about the same time the disease disappeared entirely from the population. Two groups of twenty-five mice each were given the culture in a dose of 420 organisms per mouse. The results are shown in Text-fig. 10 and Table IV.

The mortality of each group is seen to approximate closely the figures obtained from the standard control curve. This culture, therefore, is considered to be of the same virulence as Strain 1 and all cultures previously tested.

The precision of the virulence titrations is ascribed to the fact that the experimental conditions were arranged so as to eliminate as many disturbing, unknown factors as possible, and to reproduce as nearly as possible natural conditions of infection. Hence the conclusion

seems justified that the virulence of type-pure mouse strains of Friedländer-like bacilli, with which we have dealt, remains constant under natural conditions.² Mucoid strains kept type-pure on agar do not

² Dr. Gowen analyzed the results of these virulence titrations and commented upon them as follows: "The results of Tests 1 to 7 inclusive may be analyzed for the influence of period in the epidemic at which the cultures were isolated in relation to death rate. This analysis is complicated by the effect of variation in dosage. There are at least two culture tests made for each group which will give some appreciation of this factor, however. Cultures before peak of epidemic were 1, 4, 11, and 12; at peak 6, 13, 15, 16, 17, 18, 19, 32, and 33, and in the inter-epidemic period were 34 and 50. The material is set out below:

Variance	Degrees of freedom	Sum of square	Mean square
Between tests	18	2990	166.1
Within tests	19	1384	72.8
Total.....	37	4374	$Z = .412$ where for $P = .05$ $Z = .43$

"The variation between tests is greater than that within tests, as in the previous results for Strain 1. The significance of this difference is questionable, however. $Z =$ but .41 while Z for $P = .05 = .43$. Furthermore, the variation between tests does not show the consistent relation when plotted against dosage of bacteria, that was previously shown for Strain 1. In fact, the death rates appear to be quite random so far as dosage is concerned.

"Two of the 18 degrees of freedom between tests are due to the point in the epidemic where the bacilli were isolated as indicated above. Dividing the data thus the variance and its associated constants are:

Variance	Degrees of freedom	Sum of square	Mean square
Between epidemic points	2	150	75.0
Within epidemic points	16	2840	177.5
Total.....	18	2990	$Z = .431$ where for $P = .05$ $Z = 1.48$

"The analysis of the data on the epidemic point of bacterial isolation shows that the variation between epidemic points is much less than that within the different groups. The conclusion is consequently justified that the epidemic point of isolation of the bacteria plays no part in the total fatality of the epidemic it initiates in the Friedländer disease of mice. The differences within and between epidemic point groups are not significantly differentiated since $Z = .43$ while for $P = .05$ Z must be at least 1.48.

"The rapidity with which the organism kills for those animals dying offers another measure of the possible effect of period in the epidemic during which the organism was isolated. For the three groups as utilized above the average duration of life for those dying was for the preepidemic peak $8.14 \pm .26$;* peak of epi-

* Because of the skewness of the distributions these probable errors lose much of their meaning and must be interpreted with caution.

change in pathogenicity; strains obtained from animals before, during, and after epidemic periods, and in interepidemic times all agree in pathogenic activity, and strains taken from healthy carriers, from individuals acutely ill, and from animals dying of pneumonia and septicemia, are of the same high virulence. On the other hand, rough strains, developed in the laboratory, exhibit much lower virulence. The rough variant types were not observed to occur spontaneously at any phase of the natural infection.

DISCUSSION.

Throughout the studies of spontaneous Friedländer-like bacillus infection among special mouse populations, the conditions of experimentation were so controlled as to permit an analysis of the main factors determining the amount and spread of the disease. The cultures of Friedländer-like bacilli used in the present study were obtained from mice in these special populations, during various epidemic and endemic phases of the infection.

Hence, special attention is directed to the virulence titrations recorded in this paper, since they show that strains of bacilli derived from mice at epidemic and postepidemic periods, are of one type, of uniform and constant virulence, and in these respects conform to the observations made in earlier studies on mouse paratyphoid (1, *c*) and enteritidis (1, *a*) infections, and rabbit *Pasteurella* (1, *f*) infection.

The results of virulence determinations reported by us are not in

demographic group $8.11 \pm .14$; and interepidemic group $6.14 \pm .12$. Clearly there is a difference between the duration of life of the third and remaining groups. The bacteria of the interepidemic period appear to kill more rapidly and with less variation in time than those in the preepidemic or peak epidemic periods. The drawing of this conclusion does not appear to be justified, however, for if the data be examined, a clear association between the experimental groups is noted when these groups are arranged by dates. This association is largely due to the relatively long time which was taken to kill on the date of the first experiment and the short duration of life for the last two experiments. When the influence of the factor of time of experiment is removed the differences between the three groups of bacteria in the time with which they kill become insignificant, or there is no detectable influence of time of isolation of the bacteria and the duration of life for those mice which die."

accord with the views expressed by Topley, Lockhart, and Greenwood, in their papers on experimental epidemiology. These authors, it is true, have discussed the subject of virulence rather from the theoretical than from the experimental point of view, since they report few actual titrations of the mouse typhoid organisms with which they deal.

In order to make this divergence of attitude clear, we venture to review the papers of Topley and his associates at some length.

In 1919 (18), a series of tests of virulence, without control observations, is reported by Topley, in which mice of varied breeds, without known history affecting age, food, surroundings, and exposure to infection, were employed. Small numbers, all kept together in one cage, were used for each test. The culture employed was a stock strain of Pasteur Institute Danysz bacillus, fed on bits of bread. Survival of the animals following this procedure was, for the most part, random and unpredictable, and yet the varied effects were taken as indicating fluctuations in microbic virulence taking place during the experiments. No other possible factor seems to have been considered, and the results were cited as evidence in favor of the thesis stated in the earlier paragraphs of Topley's paper, that "an increase in the pathogenicity of the specific parasite is an essential factor in the rise of epidemics" (18).

That this view was premature and not conclusive is indicated in a later paper by Topley's associate, Lockhart (19), who states that "these experiments (Topley's) were not, however, especially designed to permit the observation of variations in bacterial virulence, other conditions being kept constant, and the conclusions based upon them must, therefore, be regarded as purely inferential and tentative" (page 50).

As, however, Lockhart did not control the host factor in his own tests, and remarks, with reference to his own test inoculations by way of the natural mouth portal of entry, "It is quite certain that, using any number of mice between 20 and 50, the form of the (mortality) curve will vary widely and in a random manner" (page 60), he resorted to intraperitoneal inoculations. This procedure, although useful in some ways, throws no light on the behavior of host and microbe under natural conditions. (Lange's recent experiments also demonstrate this (21).) Four of five of Lockhart's attempts to increase virulence by animal passage were reported as negative; nevertheless he states that while he has "not succeeded in controlling, or varying at will, the virulence of a given strain of *B. aertrycke*" (page 82), he does regard his tests as showing that "fluctuations in the virulence of *B. aertrycke* certainly occur" (page 81).

Another of Topley's collaborators, Wilson (20), later confirmed a view held by us in stating that there is no experimental evidence to support the hypothesis of a geometric rise and fall of bacterial virulence.

In other words, the point of view which has grown out of our own experiments (1, *a*, 1, *c*, 1, *f*, and the present paper) and the latest views of Topley's collaborators are coming more and more nearly to agree with each other.

Turning again to the results reported in the present paper, one may say that when the conditions of host and pathogenic organism are adequately controlled, it is found that mice react to the Friedländer-like bacilli in the same manner as has been stated to occur with other native animal infections,—that is, they do actually differ in respect to their native and acquired ability to resist infection (1, *g*). In the case of the Friedländer-like bacillus infection, as in the instances of other mouse and certain rabbit infectious diseases, we regard the several kinds of clinical manifestations—the septicemic, pulmonary, local or nasal infections, and the carrier state—not as sharply separated clinical conditions, but altogether as the sum total of the disease, each particular manifestation of which representing the momentary degree of resisting power of individual animals affected.

SUMMARY AND CONCLUSIONS.

A spontaneous respiratory disease of mice incited by Friedländer-like bacilli has been described. The bacilli inducing the disease, while morphologically and culturally indistinguishable from the usual varieties of Friedländer bacilli, are antigenically distinct from the common type strains known. The bacilli grow better in cultures at 23°C. than at 37°C.

The disease in mice displayed an incubation period of about 48 hours. About 50 per cent of an exposed population succumbed to septicemic and acute hemorrhagic, pneumonic processes. Among the exposed animals were some individuals which remained apparently well and carried the pathogenic bacilli in their nasal passages.

The several manifestations of the spontaneous disease were reproduced by instilling small numbers of the cultured bacilli into the nasal passages. 48 hours after inoculation, certain mice had already succumbed; the deaths continued to occur, so that by the end of the 2nd week, 70–80 per cent of the animals had died. Among the survivors certain carriers of the bacilli in the nasal passages occurred; a few appeared entirely refractory to the infection. The succumbing

mice showed at autopsy and by culture septicemia and diffuse pneumonic inflammation.

No fluctuation in virulence could be detected in bacilli derived from mice while the disease was spreading spontaneously at the periods of epidemic rise, interepidemic interval, or postepidemic quiescence. Moreover, the bacilli cultured from the nares of apparently healthy carriers were equally pathogenic with those taken from the blood and lungs of animals succumbing quickly.

No rough colony variants were cultured at any phase of the spontaneous disease, although they were readily obtainable by artificial culture. The variant strains proved stable and of low virulence.

This Friedländer bacilli infection in mice takes several clinical courses, depending on variations in host reaction and not depending on bacterial variation. The particular type of infection manifested is determined by the degree of resisting power displayed by the infected animals at the moment that the infection occurred and progressed.

We desire to thank Dr. L. Julianelle for his kindness in titrating the mouse Friedländer strains in his type sera, and Dr. John W. Gowen for analyzing mathematically the experimental data on which a part of this paper is based.

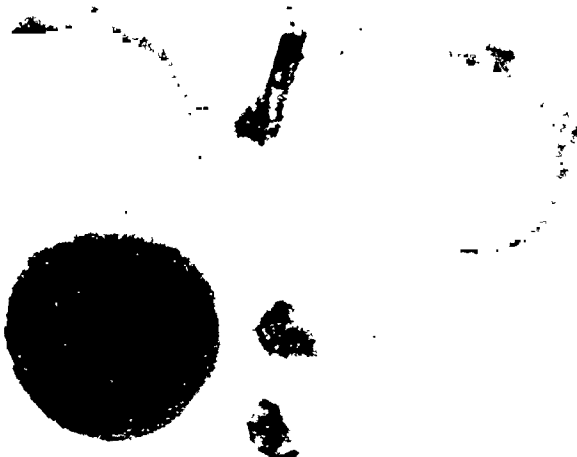
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EXPLANATION OF PLATE 33.

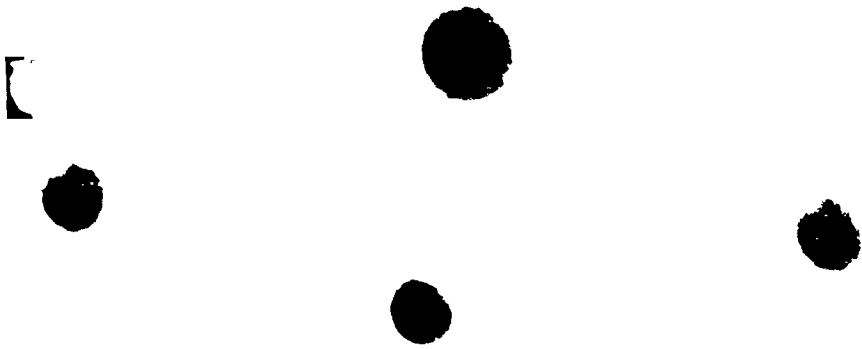
FIGS. 1-3. Mouse strains of Friedländer's bacillus. Smooth and rough colonies. $\times 5$.



1



2



3

IMMUNOLOGICAL STUDIES IN RELATION TO THE SUPRARENAL GLAND.

I. HEMOLYSIN FORMATION IN NORMAL RATS.

BY J. MARMORSTON-GOTTESMAN, M.D., AND DAVID PERLA, M.D.

(From the Laboratory Division of Montefiore Hospital, New York.)

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In the course of studies in this laboratory on the effect of suprarenal-ectomy on antibody formation, it became necessary to determine the hemolysin formation in normal rats. The hemolysin formation in several animals, particularly the rabbit, has been thoroughly investigated, but few studies on hemolysin formation in rats have been reported. In relation to a study of the effect of x-ray on antibody formation, Hektoen (1) produced fairly high hemolysin titers in rats with a small single intraperitoneal injection of sheep cells. Rats present certain peculiarities in immunity phenomena which make such studies of interest. A normal rat produces relatively slight precipitin (2). A state of local hypersensitiveness cannot be induced by injections of a foreign protein (2). Anaphylactic shock is produced with some difficulty (3). Rats withstand huge quantities of various poisons and toxins such as diphtheria (6), tetanus toxin (7) and histamine (4, 5).

Method.

The blood of the sheep was received in a preserving fluid recommended by Rous and Turner (8), consisting of isotonic solutions of glucose and sodium citrate. Cells were never kept longer than 6 days in this fluid and control daily fragility tests were employed. The cells used for injection and testing were washed three times with physiological salt solution to which was added .1 per cent of calcium chloride as recommended by Snapper (9) to prevent a disturbance of the osmotic equilibrium between the red blood cells and the saline solution. Single and multiple injections of varying quantities of antigen were given intraperitoneally and comparative studies of the hemolysin formation were made. Blood was obtained at intervals from the rats by puncturing the heart and withdrawing 1 to 2 cc. of blood. It was necessary to fix the rat on a board specially constructed and to

introduce the needle close to the sternum over the area of cardiac impulse. Repeated punctures at one sitting increase the dangers of fatal hemorrhage, but with practice, fatalities were reduced to less than 5 per cent.

In determining the titer of hemolysin present in the serum, progressive dilutions of the rat serum to be tested were made. .1 cc. of a 5 per cent suspension of sheep cells and $2\frac{1}{2}$ units of complement were added. The total volume of each tube was brought up to 1 cc. with physiological saline. The tubes were incubated at 37° during 30 minutes and readings were made immediately. Traces of hemolysis equivalent to a one plus or lesser reading on a scale in which four plus represents complete hemolysis, were disregarded. That dilution in which hemolysis was partial but definite, that is, a two plus reaction, was read as the titer of the hemolysin present in the serum.

Hemolysin Formation after a Single Intraperitoneal Injection of Sheep Cells.

Twenty adult albino rats raised in the laboratory, from Wistar Institute stock, and all of approximately the same size and age, were used in this series. 1 cc. of a 10 per cent suspension of washed sheep cells was injected intraperitoneally into each rat. The titer was first determined 5 days after the injection and subsequent determinations were made on the 8th, 11th and 14th days. As noted in Table I, the height of the antibody formation is reached on or about the 5th day. The titer then gradually drops and is either very low or disappears entirely 14 days after the injection. The hemolysin titer in normal adult rats injected with 1 cc. of a 10 per cent suspension of sheep cells varies from 1:4000 to 1:24,000. Occasionally a titer of 1:2000 or 1:1600 is noted and in some rats the titer falls below this. The variation is considerable but it is not as striking as in rabbits. Although a high titer is reached with one injection of a small quantity of red blood cells, it is not maintained. The titer drops from 1:16,000 on the 5th day to 1:4000 or 1:2000 on the 8th day, and rapidly to 1:800 or 1:400 on the 11th day, while by the 14th day, it is about 1:200. The average initial titer of the twenty rats was 1:7000 on the 5th day; on the 8th day, the average titer was 1:3270; on the 11th, it was 1:580 and on the 14th day, the average titer was 1:200, although the titer in several animals had practically disappeared at this time.

A single injection of ten times this amount or 1 cc. of undiluted red blood cells was given to eight rats. It is seen from Table II that in these instances no titer exceeded 1:4000 and many titers were much

lower. The average initial titer in the rats in our series was 1:2000 after 5 days as compared with the average titer of 1:7000 of the previous series. The second and third readings were correspondingly lower (see Figs. 1 and 2).

Single injections of .5 cc. of a 10 per cent suspension had the same effect on hemolysin formation as 1 cc. of a 10 per cent suspension.

TABLE I.

Hemolysin Formation in Normal Rats, Each Receiving a Single Intraperitoneal Injection of 1 Cc. of a 10 Per Cent Suspension of Washed Sheep Cells and Tested at Intervals of 5, 8, 11 and 14 Days after the Injection.

Rat No.	Titer			
	5th day	8th day	11th day	14th day
61	6,000	2,000	600	100
62	2,000	200	160	200
63	24,000	8,000	1,600	600
64	2,000	2,000	200	80
65	32,000	16,000	2,000	600
66	3,000	2,000	160	150
27	4,000	Died		
30	300	160	10	20
88	8,000	800	1,000	300
89 A	12,000	1,000	800	100
103 A	1,600	Died		
104 B	400	1,000	80	
105	8,000	2,000	200	
134	1,600	1,600	600	
135	1,000	800	400	200
154	1,000	800	400	160
16	4,000	1,600	600	80
17	12,000	800	600	
23	16,000	2,000		
22	800	160		

However, with injections of .2 cc. of a 10 per cent suspension of sheep cells, antibody titers were considerably less than with 1 cc. of the dilution, although not in mathematical proportion. With single injections of .05 cc. of a 10 per cent suspension or one-twentieth the amount first used, the hemolysin titer rose to a maximum height of 1:800. As will be noted in Table III, the antibody formation in many

TABLE II.

Hemolysin Formation in Normal Rats, Each Receiving a Single Intraperitoneal Injection of 1 Cc. of Undiluted Washed Sheep Cells and Tested at Intervals of 5, 8, 11 and 14 Days after the Injection.

Rat No.	Titer			
	5th day	8th day	11th day	14th day
139	4,000	600	400	30
140	4,000	2,000	800	80
141	1,600	400	160	
142	1,600	1,600	800	200
143	800	2,000	2,000	
144	1,600	200	400	
151	1,000	1,000	600	400
152	1,600	1,600		
153	2,000	600	—	400

TABLE III.

Hemolysin Formation in Normal Rats, Each Receiving a Single Intraperitoneal Injection of Small Amounts of a 10 Per Cent Suspension of Washed Sheep Cells and Tested at Intervals of 5, 8 and 11 Days after the Injection.

Rat No.	Amount injected 10 per cent suspension cc.	Titer		
		5th day	8th day	11th day
90	0.2	400	100	100
94	0.2	2,000	2,000	400
95	0.2	1,600	1,000	800
91	0.1	2,000	2,000	400
96	0.1	1,000	1,000	400
97	0.1	800	1,000	800
92	0.05	200	Died	
93	0.05	800	0	0
98	0.05	1,000	400	Died
136	0.05	400	400	30
137	0.05	400	400	80
138	0.05	200	400	160

instances was approximately one-twentieth as high as with twenty times the quantity of injected antigen. The relative curves with these varying amounts of antigen were similar, the titer disappearing on or about the 14th day (see Fig. 1).

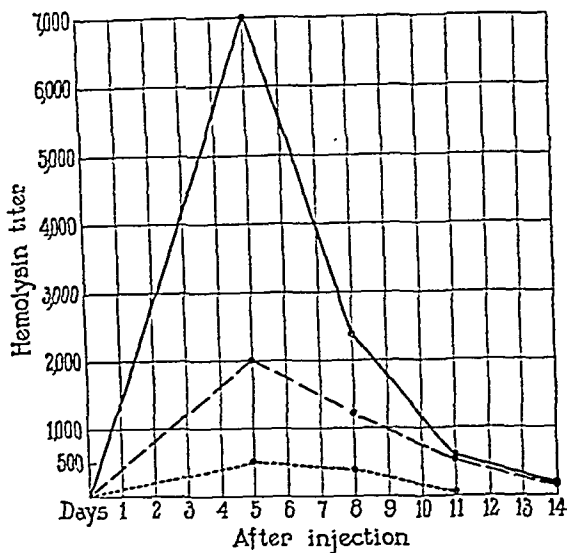


FIG. 1. Curves of average hemolysin titers with varying quantities of antigen.
 ————— Curve of average hemolysin titer of 20 normal rats injected with 1 cc. of a 10 per cent suspension of sheep cells.
 - - - Curve of average hemolysin titer of 9 normal rats injected with 1 cc. of undiluted sheep cells.
 Curve of average hemolysin titer of 6 normal rats injected with 0.5 cc. of a 10 per cent suspension of sheep cells.

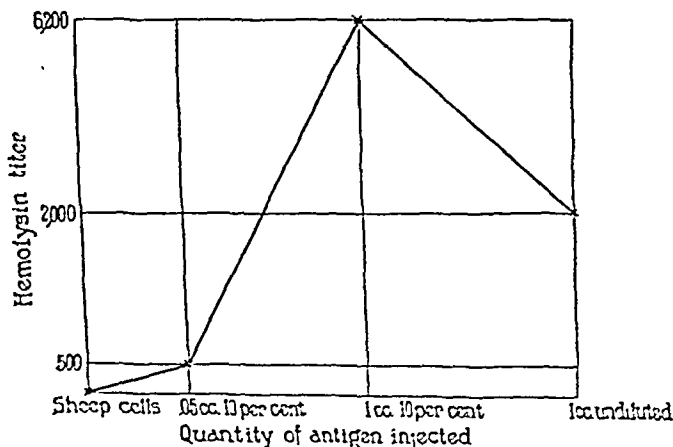


FIG. 2. Antigen-antibody curve in 35 normal rats injected intraperitoneally with varying quantities of sheep cells. The hemolysin titer in each instance is the average optimum titer obtained in each series.

From these experiments, it may be concluded that in normal adult albino rats, relatively high hemolysin titers may be obtained with a single intraperitoneal injection of a small quantity of sheep cells. There is, however, an optimum amount, 1 cc. of a 10 per cent suspension, which produces a definitely high titer. This titer rapidly drops and practically disappears from the blood after a period of 14 to 18 days. Amounts greater than this are not only ineffective in increasing the antibody formation but are followed by lower titers than those obtained with one-tenth the quantity of antigen. Much smaller

TABLE IV.

Hemolysin Formation in Normal Rats, Each Receiving Three Intraperitoneal Injections of Varying Amounts of Washed Sheep Cells at Varying Intervals and Tested 5, 8, 11 and 14 Days after the Last Injection.

Rat No.	No. of injections	Amount of each injection	Interval between injections	Titer			
				5th day	8th day	11th day	14th day
			<i>days</i>				
145	3	1 cc. 1/10	1	2,000	1,600	400	200
146	3	1 " 1/10	1	16,000	1,600	800	400
147	3	1 " 1/10	1	1,000	400	160	100
148	3	1 " undiluted	1	2,000	1,600	400	20
149	3	1 " "	1	1,000	400	30	40
150	3	1 " "	1	600	Died		
18	3	1 " 1/10	5	160	160	300	40
19	3	1 " 1/10	5	100	200	Died	
20	3	1 " 1/10	3	4,000	400	300	200
21	3	1 " 1/10	3	4,000	160	100	100

amounts produce correspondingly lower titers although the ratio is not always mathematically proportional.

Repeated Injections of Red Blood Cells.

Three injections each of 1 cc. of a 10 per cent suspension of sheep cells were given to three rats at daily intervals. The hemolysin titer was tested on the 5th, 8th, 11th and 14th days following the last injection. From Table IV it is seen that the antibody titer tested on the 5th day after the last injection is less than that resulting from a single injection of antigen. The first test was made on the 8th day following the *first* injection of antigen, and if Table IV is compared

with Table I, it is noted that the titer 5 days after the last of three injections repeated at daily intervals with 1 cc. of a 10 per cent suspension is about the same as on the 8th day following a single injection of 1 cc. of a 10 per cent suspension. It would seem that repeated injections at daily intervals failed to increase the antibody formation.

TABLE V.

Hemolysin Formation in Normal Rats, Each Receiving Three Intraperitoneal Injections of a 10 Per Cent Suspension of Washed Sheep Cells at 5 Day Intervals.

Rat No.	No of injections	Date of injection	Amount injected 10 per cent suspension	Date tested	Titer
			cc.		
1	3	9/ 7/27	0.5	9/ 7/27	0*
		9/12/27	0.5	9/12/27	8,000
		9/17/27	0.5	9/17/27	200
				9/22/27	10
				9/26/27	0
2	3	9/ 7/27	0.5	9/ 7/27	0*
		9/12/27	0.5	9/12/27	10,000
		9/17/27	0.5	9/17/27	200
				9/22/27	0
3	3	9/ 7/27	0.5	9/ 7/27	0*
		9/12/27	0.5	9/12/27	16,000
		9/17/27	1.0	9/17/27	200
				9/22/27	40
				9/26/27	10
				10/ 1/27	20
4	3	9/ 7/27	0.5	9/ 7/27	0*
		9/12/27	0.5	9/12/27	10,000
		9/17/27	1.0	9/17/27	2,000
				9/19/27	200
				9/22/27	20
				9/26/27	10

*Sera tested before first injection contained no hemolysin.

Three injections at intervals of 3 days, each of 1 cc. of a 10 per cent suspension of red blood cells were given to two rats. The hemolysin titer was determined on the 3rd, 6th, 9th and 12th days following the last injection. Here again, it is noted, from Table IV, that the antibody titer was only slightly less than with a single injection.

Repeated injections at 5 day intervals, each of .5 cc. of a 10 per cent suspension for three injections, were given to several rats. The antibody titer was determined before each injection. From Table V it is seen that in spite of repeated injections of .5 cc. of a 10 per cent suspension of red blood cells, the titer never exceeded the initial rise which it reached 5 days after the first injection. Furthermore, in spite of repeated injections, the titer dropped progressively exactly as in the instances with a single injection. Subsequent injections had practically no effect on the curve of antibody formation produced by the first injection.

Repeated injections of similar amounts of antigen at daily intervals, or at intervals of 3 or 5 days, do not increase the antibody titer nor do repeated subsequent injections of the same amount of antigen modify the curve of hemolysin formation resulting from the first injection.

DISCUSSION.

The curve of the hemolysin titer in healthy rabbits has been carefully studied by Bulloch (10), Sachs (11), Lüdke (12) and Remy (13). Following single or multiple injections of sheep cells, Bessau and Paetsch (14) and Sachs (11) observed a negative phase during which no antibodies were detected in the serum. The length of this negative phase was observed by Sachs to be entirely independent of the amount of blood cells injected, but varied with the method of injection. Following the subcutaneous route, this period was generally 7 days, but in instances in which the intravenous or intraperitoneal route was used, this latent period was reduced to 3 days (Bulloch) or to 2 to 4 days (Sachs). The hemolysin titer then rose to a maximum on the 5th to the 6th day where it was maintained for 24 hours after which it gradually fell. Wolf (15) observed a second rise in rabbits on the 7th to the 10th day. The titer then gradually dropped. There was no direct proportion between the amount of antigen injected and the antibody titer which was produced. Sachs studied the relation of amboceptor to red blood cells and complement in rabbits and found that the appearance of the immune body coincided with the disappearance of the foreign red blood cells from the circulation of the immune animal. Lüdke (16) found that ox cells injected into rabbits subcutaneously completely disappeared in 8 days from the circulation, when hemolysin could first be detected. Probably the immune substances first formed by the injection of the antigen hemolyzed the red blood cells present in the circulation by specific action. In this process complement was also utilized. With the neutralization of the antigen present, the amboceptor became detectable.

In our work we have found that repeated injections of small amounts of red blood cells did not increase the titer of the hemolysin formed by a single injection nor did subsequent injections maintain the titer of hemolysin produced by the first injection. Possibly the amounts injected after the first injection were hemolyzed by existing amboceptor in the serum of the immunized animal and were therefore unable to stimulate further antibody production.

Coca (17) found that after a single injection of 1 cc. of a 10 per cent suspension of sheep cells in rabbits, average titers 1 week after injection reached a maximum of 1:250 (.004). When twenty times this quantity was used, the titer obtained was 1:154 (.0065), definitely lower than with the larger amounts. In rats, we found a similar antibody-antigen relation; with ten times the quantity of antigen, the amboceptor titer dropped. Injections of smaller quantities of antigen in rabbits, Coca found, were more effective in producing a high titer than injections of larger amounts. The observations of Coca and others have shown, however, that in rabbits the number of injections and the intervals between injections influence the hemolysin titer. In rats, the titer following the initial injection of sheep cells was not raised by repeated injections of the same amount of sheep cells. Further subsequent injections had little effect on the curve of the hemolysin formation initiated by the first injection.

SUMMARY AND CONCLUSION.

Hemolysin formation was studied in normal rats. It was found that a single intraperitoneal injection of 1 cc. of a 10 per cent suspension of red blood cells results in an optimum high hemolysin titer 5 days after injection. The titer gradually falls, the hemolysin disappearing from 14 to 18 days after the injection. Larger amounts are less effective in the production of amboceptor. Much smaller amounts produce correspondingly lower hemolysin titers, although the ratio is not mathematically proportional. Repeated injections of 1 cc. of a 10 per cent suspension of red blood cells at daily intervals or at intervals of 3 or 5 days for three injections do not increase the hemolysin titer over that resulting from a single injection. Furthermore, the curve of antibody formation following a single small intraperitoneal injection of red blood cells is not altered by subsequent injections of similar amounts.

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IMMUNOLOGICAL STUDIES IN RELATION TO THE SUPRARENAL GLAND.

II. HEMOLYSIN FORMATION IN SUPRARENALECTOMIZED RATS.

BY DAVID PERLA, M.D., AND J. MARMORSTON-GOTTESMAN, M.D.

(From the Laboratory Division of Montefiore Hospital, New York.)

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There is a great deal of evidence both from morphological and physiological studies that the suprarenal gland plays a significant rôle in the defensive mechanism of the body against intoxication. During the past 7 years numerous observers have established that the resistance of suprarenalectomized rats and rabbits to trauma and to injections of various poisons and toxins is markedly diminished, particularly during the first weeks following the operation. However, only a few studies on the antibody capacity of animals following bilateral suprarenalectomy have been reported. The literature bearing on the relation of the suprarenal gland to the formation of antibodies has been reviewed by Také and Marine (1). Experiments dealing with the antibody capacity of animals following bilateral suprarenalectomy have been limited because of technical difficulties. The most suitable laboratory animals are the rabbit and the rat since a high percentage of these animals survive bilateral suprarenalectomy. Také and Marine found that bilaterally suprarenalectomized rabbits show hemolysin titers distinctly higher than the control animals. Jaffé and Marine (2) observed that rats repeatedly injected with typhoid vaccine 3 weeks following bilateral suprarenalectomy developed agglutinin titers slightly higher than normal control rats. The evidence presented in the literature, however, is of such a contradictory nature that further studies in this field seemed desirable.

The rat was chosen as the experimental animal as over 85 per cent of rats survive bilateral suprarenalectomy in good condition and these present a state of suprarenal insufficiency for a considerable period of

time. The presence of accessory cortical tissue accounts for the recovery and compensation in these animals.

In a preliminary study on the hemolysin formation in normal rats, it was found that high titers were obtained following a single intraperitoneal injection of a small amount of sheep cells (1 cc. of a 10 per cent suspension). This rapid method of immunization, therefore, is definitely advantageous in studying the early period after bilateral suprarenalectomy. The titer was determined at intervals of 5, 8, 11 and 14 days after injection.

Methods.

The methods used have been described in a previous communication (3). Adult albino rats raised in our laboratory, from Wistar Institute stock, were used. The rats were all about 5 months old and of approximately the same weight. The suprarenal glands were removed at a single operation through the posterior route in the manner described by Jaffe (4).^{*} Over 85 per cent of our rats survived 4 weeks or longer after the operation. All rats were carefully autopsied after the experiments and the operated areas were examined, the presence or absence of gross accessory suprarenal tissue determined and the weight of the thymus recorded. Blood for testing was drawn from the heart by puncture.

In the first group of experiments to be reported, we studied the influence of varying the time interval following suprarenalectomy on the titer, keeping the amount of antigen injected constant. In the second group, the quantity of antigen injected was varied, but the time interval kept constant. In the first part of the work, all rats received a single intraperitoneal injection of 1 cc. of a 10 per cent suspension of sheep cells 2, 7, 14 and 28 days following the operation. A small series of unilaterally nephrectomized rats injected 2 weeks after the operation was tested as a control for operative trauma. In the second group of experiments, two series of suprarenalectomized rats were injected with .05 cc. of a 10 per cent suspension of sheep cells and 1 cc. undiluted sheep cells respectively, 2 weeks after the operation. In preliminary experiments, the range of hemolysin titers in normal rats had been determined with these amounts. Normal rats, and rats in which the perisuprarenal tissue was torn, were tested in both series.

Twenty normal rats were injected with 1 cc. of a 10 per cent suspension of sheep cells and tested 5, 8, 11 and 14 days after the injection. It was found that with this amount, the titer was above 1:2000 in over 70 per cent of the rats. A few rats showed low titers.¹ The average titer of these twenty normals was 1:7000.

^{*} Ether anesthesia was used in all operative procedures.

Hemolysin Formation in Bilaterally Suprarenalectomized Rats Receiving a Single Intraperitoneal Injection of 1 Cc. of a 10 Per Cent Suspension of Sheep Cells 48 Hours after Operation.

Fourteen rats were used in the first series. Of these, six were bilaterally suprarenalectomized, six were normal and two were traumatized by tearing the perisuprarenal tissue. All were injected

TABLE I.

Hemolysin Formation in Bilaterally Suprarenalectomized Rats, Intraperitoneally Injected 48 Hours after the Operation with 1 Cc. of 10 Per Cent Suspension of Washed Sheep Cells and Tested 5, 8, 11 and 14 Days after Injection.

Rat No.	Operation	Interval between operation and injection	Amount injected	Titer				Weight at operation	Weight at death	Weight of thymus
				5th day	8th day	11th day	14th day			
		days						gms.	gms.	mg.
41	Suprarenalectomy	2	1 cc. 1/10	6,000	4,000	800	Dead	160	158	178
42	"	"	"	400	1,600	Dead		150	190	185
43	"	"	"	100	400	80		175	166	326
44	"	"	"	200	400	400	300	165	185	Large
45	"	"	"	16,000	8,000	400	160	200	180	320
46	"	"	"	100	800	20	10	225	220	Small
59	Traumatization	"	"	16,000	10,000	3,000	0?	160	165	162
60	"	"	"	800	800	400	160	150	150	198
61	Control	—	"	6,000	2,000	600	100	183		
62	"	—	"	2,000	600	160	200	193		
63	"	—	"	24,000	8,000	1,600	600	184		
64	"	—	"	2,000	2,000	200	80	170		
65	"	—	"	16,000	32,000	2,000	600	178		
66	"	—	"	3,000	2,000	160	160	185		

intraperitoneally with 1 cc. of a 10 per cent suspension of sheep cells 48 hours after the operation. Tests were made at intervals of 5, 8, 11 and 14 days. As will be noted in Table I the titers of the suprarenalectomized rats were markedly lower than those of the normal rats. 80 per cent of the suprarenalectomized rats showed an initial titer of 1:400 or less. Two suprarenalectomized rats showed a normal

titer. A comparison between the titer obtained in normal rats¹ and that found in rats following suprarenalectomy reveals a marked depression in hemolysin titer 48 hours after suprarenalectomy. If one examines the curve of antibody formation in those rats showing a low titer, it will be noted that there is a subsequent rise in hemolysin titer at the second reading, the peak of the curve being reached on the 8th instead of the 5th day, as in the normal rats. The depression of antibody formation during the 1st week coincides with the marked depression in resistance of suprarenalectomized rats noted by numerous observers (2, 5-7). It was found by one of us (8, 9) that the height of susceptibility of rats to typhoid and to histamine poisoning is reached during the period from 5 to 7 days after bilateral suprarenalectomy.

Hemolysin Formation in Bilaterally Suprarenalectomized Rats Receiving a Single Intraperitoneal Injection of 1 Cc. of a 10 Per Cent Suspension of Sheep Cells 7 Days after Operation.

A second series of thirteen rats, nine of which were suprarenalectomized, two traumatized and two normal, was injected intraperitoneally with 1 cc. of a 10 per cent suspension of sheep cells 7 days after the operation. The titers were determined at intervals of 5, 8, 11 and 14 days after the injection. From Table II it is seen that the suprarenalectomized rats still show a definite depression in antibody formation. In two instances where the titer was 1:2000 and 1:3000 a fragment of suprarenal tissue had been left behind. These two instances are therefore excluded from consideration in the series. In this series, 40 per cent gave initial titers below 1:400, 85 per cent below 1:2000 and 14 per cent gave titers of 1:2000 to 1:3000 or within the low titer range of normal rats. The average titer 7 days after the operation was 1:1070 or approximately one-seventh that of normal rats. These figures are slightly higher than those of Series 1, but they still show a definite depression in the hemolysin titer. It is obvious that the suprarenalectomized rats still yield definitely lower antibody titers when the rats were injected with small amounts of sheep cells 7 days as well as 48 hours after the operation. The titers

¹ See Table I of Paper I (3).

of the traumatized animals are likewise depressed. This observation will be discussed elsewhere in this paper.

TABLE II.

Hemolysin Formation in Bilaterally Suprarenalectomized Rats, Intraperitoneally Injected 7 Days after the Operation with 1 Cc. of 10 Per Cent Suspension of Washed Sheep Cells and Tested 5, 8, 11 and 14 Days after Injection.

Rat No.	Operation	Interval between operation and injection	Amount injected	Titer				Weight at operation	Weight at death	Weight of thymus
				5th day	8th day	11th day	14th day			
		days						gms.	gm.	mg.
40	*Incomplete suprarenal-ectomy	7	1 cc. 1/10	2,000	800	10	10	180	195	370
32	Suprarenalectomy	"	"	1,500	1,000	300	200	170	145	375
33	"	"	"	1,600	80	60	Dead	190	180	365
34	"	"	"	900	160	60	40	160	150	298
35	"	"	"	3,000	Dead			160	145	402
13	"	"	"	200	20	20		228		
14	"	"	"	200	100	20		195		
15	"	"	"	100	100	80		200		
38	*Incomplete suprarenal-ectomy	"	"	3,000	800	Dead		275	255	Large
36	Traumatization	"	"	1,800	3,000	200	40	175	175	273
37	"	"	"	800	800	40	80	190	183	223
27	Control	—	"	4,000	Dead			300	300	230
30	"	—	"	300	160	40		250		

*Small fragment of right left *in situ*.

Hemolysin Formation in Bilaterally Suprarenalectomized Rats Receiving a Single Intraperitoneal Injection of 1 Cc. of a 10 Per Cent Suspension of Sheep Cells 14 Days after Operation.

A third series of twelve rats, eight of which were suprarenalectomized, two traumatized and two normal, was injected intraperitoneally with 1 cc. of a 10 per cent suspension of sheep cells 14 days after the operation and tested 5, 8, 11 and 14 days after the injection. From

Table III it is evident that the antibody titer was higher than in the series injected 7 days after the operation. In only one instance was the initial titer 1:800, and five animals or 60 per cent showed a titer above 1:2000. The average titer was 1:2770 or about one-half the titer of the normal rats. Several of the titers of the operated animals reached the lower normal range. It is interesting that after the 2nd

TABLE III.

Hemolysin Formation in Bilaterally Suprarenalectomized Rats, Intraperitoneally Injected 14 Days after the Operation with 1 Cc. of 10 Per Cent Suspension of Washed Sheep Cells and Tested 5, 8, 11 and 14 Days after Injection.

Rat No.	Operation	Interval between operation and injection	Amount injected	Titer				Weight at operation	Weight at death	Weight of thymus
				5th day	8th day	11th day	14th day			
		days						gm.	gm.	mg.
70	Suprarenalectomy	14	1 cc. 1/10	3,000	Dead			160	150	175
71	"	"	"	1,600	400	300	Dead	168	145	200
72	"	"	"	3,000	Dead			178	178	184
73	"	"	"		8,000	400		240	160	206
74	"	"	"	1,000	800	800	0	260	260	416
80	*Incomplete suprarenalectomy	"	"	6,000	600	Dead		177	170	
81	Suprarenalectomy	"	"	2,000	1,600	600		161	157	
83	"	"	"	800	1,000	600	600	292	232	
75	Traumatization	"	"	1,000	800	1,600	20	260	270	275
76	"	"	"	160	40	20	0	222	185	295
88	Control	—	"	8,000	800	800	300	148	145	275
89 A	"	—	"	12,000	1,000	800	100	200	220	295

*Small fragment left *in situ*.

week, the capacity to form antibodies in response to injections of small amounts of sheep cells definitely rises, but is still somewhat below that of normal rats. As has been established by Jaffe (4) and Marine, there is a marked hyperplasia of the thymus and the lymph nodes 2 weeks after suprarenalectomy. This may be a factor in the explanation of the compensatory rise in antibody formation at this time.

Hemolysin Formation in Bilaterally Suprarenalectomized Rats Receiving a Single Intraperitoneal Injection of 1 Cc. of a 10 Per Cent Suspension of Sheep Cells 4 Weeks after Operation.

A fourth series of nine rats, four of which were suprarenalectomized, two traumatized and three normal, was injected with 1 cc. of a 10 per cent suspension of sheep cells 4 weeks after operation. Two suprarenalectomized rats gave titers below 1:1000. At 4 weeks, the titer of hemolysin formation had not returned to normal but was definitely lowered. No definite comparison can be made from this small series.

TABLE IV.

Hemolysin Formation in Bilaterally Suprarenalectomized Rats, Intraperitoneally Injected 28 Days after the Operation with 1 Cc. of 10 Per Cent Suspension of Washed Sheep Cells and Tested 5, 8 and 11 Days after Injection.

Rat No.	Operation	Interval between operation and injection	Amount injected	Titer			Weight at operation	Weight at death	Weight of thymus
				5th day	8th day	11th day			
51	Suprarenalectomy	days	1 cc. 1/10	2,000	4,000	Dead	gms.	gms.	gms.
52	"	"	"	200	Dead		175	170	552
53	"	"	"	300	200	160	175	145	
55	"	"	"	1,000	Dead		140	130	313
57	Traumatization	"	"	160	200	80	220	280	185
58	"	"	"	3,000	800	160	170	—	—
103 A	Control	—	"	1,600	Dead		250	260	223
104 B	"	—	"	400	1,000	80	245	250	186
105 C	"	—	"	8,000	2,000	200	290	300	261

From these four series it may be concluded that in bilaterally suprarenalectomized rats, the hemolysin formation resulting from a single intraperitoneal injection of 1 cc. of a 10 per cent suspension of sheep cells is depressed in all periods up to and including 4 weeks following the operation. The depression is greatest during the 1st week, lessens slightly during the 2nd week and diminishes considerably during the 3rd week. After 4 weeks, the hemolysin titer is still below the normal reading.

Hemolysin Formation in Bilaterally Suprenalectomized Rats Receiving a Single Intraperitoneal Injection of .05 Cc. of a 10 per Cent Suspension of Sheep Cells 2 Weeks after Operation.

The second group of experiments was carried out to determine the effect of varying amounts of antigen on the antibody formation.

TABLE V.

Hemolysin Formation in Bilaterally Suprenalectomized Rats, Intraperitoneally Injected 14 Days after the Operation with .05 Cc. of 10 Per Cent Suspension of Washed Sheep Cells and Tested 5, 8 and 11 Days after Injection.

Rat No.	Operation	Interval between operation and injection	Amount injected	Titer			Weight at operation	Weight at death	Weight of thymus
				5th day	8th day	11th day			
103	Suprenalectomy	14	0.05 cc. 1/10	300	300	40	180	170	175
107	"	"	"	300	200	20	195	180	200
108	"	"	"	200	300	200	140	110	160
109	"	"	"	200	200	40	200	185	310
110	"	"	"	400	200	10	190	170	271
111	"	"	"	40	40	Dead	260		
112	Traumatization	"	"	200	80	"	275	300	176
113	"	"	"	40	0	"	300	305	196
114	"	"	"	20	20	10	260	260	140
136	Control	—	"	400	400	30	184		
137	"	—	"	400	400	20	225		
138	"	—	"	200	400	160	166		
92	"	—	"	200					
93	"	—	"	800	0	0			
98	"	—	"	1,000	400		155	155	

In the first series of this group, fifteen rats were used, six of which were suprarenalectomized, six normal and three traumatized by tearing the perisuprarenal tissue. All were injected 2 weeks after the operation intraperitoneally with .05 cc. of a 10 per cent suspension of sheep cells (brought up to a volume of .5 cc.). This quantity of antigen represents one-twentieth the amount used for injection

throughout the first group of experiments. It will be noted from Table V that the range of hemolysin titers in normal rats was from 1:200 to 1:1000, the average titer being 1:500. In the suprarenalectomized group, the titers ranged from 1:20 to 1:400, the average titer being 1:240 or one-half the normal. Here as in the first group of experiments, the hemolysin formation in rats injected 2 weeks after bilateral suprarenalectomy is less than that of the control rats. It is significant that as in the first group, the traumatized controls gave lower titers than either the normal or suprarenalectomized rats of this period.

Hemolysin Formation in Bilaterally Suprarenalectomized Rats Receiving a Single Intraperitoneal Injection of 1 Cc. of Undiluted Sheep Cells 2 Weeks after Operation.

In the second series of the second group eighteen rats were used, six of which were bilaterally suprarenalectomized, nine normal and three traumatized in the perisuprarenal area. All were injected 2 weeks after the operation with 1 cc. of undiluted sheep cells. In the normal rats of this series, hemolysin titers in no instance exceeded 1:4000. 65 per cent showed titers below 1:2000. These titers are strikingly lower than those found in the normal rats injected with 1 cc. of a 10 per cent suspension (one-tenth the amount).¹ The average titer in the series of rats injected with 1 cc. of undiluted sheep cells was 1:2000 as compared with 1:7000, the average titer in the normal rats receiving one-tenth the amount of antigen.

Coca (10) found that small quantities of sheep cells are more effective in the production of hemolysin in rabbits than larger amounts. There is a definite optimum antigen amount for the production of hemolysin in rats. Larger or smaller amounts are less effective.

In contrast with this drop in hemolysin titer in *normal rats* injected with 1 cc. of undiluted sheep cells, the *suprarenalectomized* series injected 2 weeks after the operation with the same amount of antigen shows a definitely higher titer than that given by the normal rats. 100 per cent gave a titer of 1:2000 or above, 90 per cent gave a titer above 1:4000 and 50 per cent, a titer above 1:8000! In the normal rats, only 40 per cent gave titers above 1:2000 and none gave titers above 1:4000. 60 per cent gave titers of 1:1600 or less. The average

titer in the suprarenalectomized animals injected with large amounts of antigen was 1:7330. Quantities of 1 cc. of a 10 per cent suspension produced slightly lower titers in suprarenalectomized rats when injected 2 weeks after the operation than in normal rats. Quantities

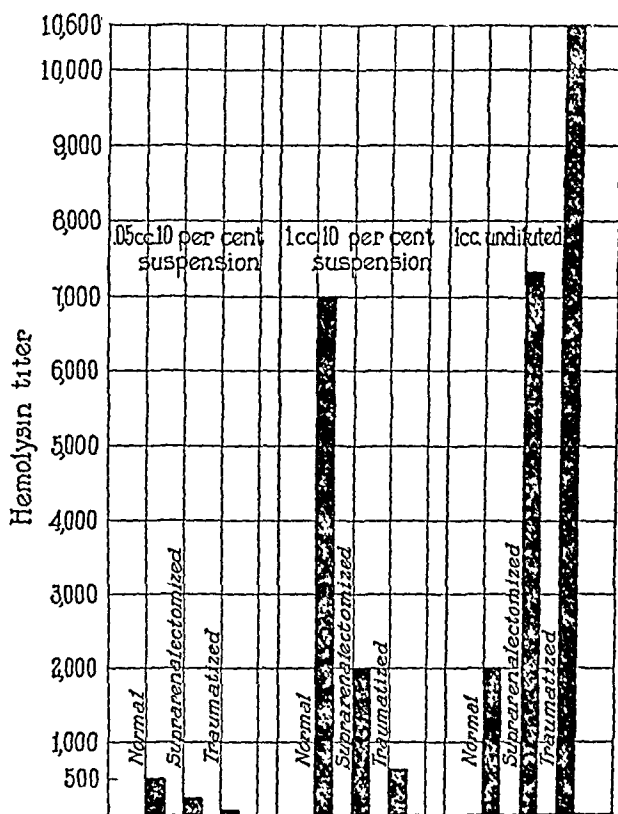


FIG. 1. The relation of hemolysin formation to the quantity of antigen injected in normal rats, in suprarenalectomized rats and in rats in which the perisuprarenal tissue had been traumatized. All rats were injected intraperitoneally 2 weeks after the operation and tested 5 days after the injection. The hemolysin titer in each group is the average optimum titer obtained in the series of rats with .05 cc. of a 10 per cent suspension, 1 cc. of a 10 per cent suspension and 1 cc. of undiluted sheep cells, respectively.

ten times this amount, however, produced titers definitely higher in the suprarenalectomized rats than in the normal rats. The optimum antigen amount necessary to produce the highest titer in suprarenal-ectomized rats injected 2 weeks after the operation, therefore, is

evidently ten times the optimum necessary to produce the highest titer in normal rats.

The difference in curves illustrating the antigen-antibody ratio can be readily observed in Fig. 1. The titers obtained in suprarenalecto-

TABLE VI.

Hemolysin Formation in Bilaterally Suprarenalectomized Rats, Intraperitoneally Injected 14 Days after the Operation with 1 Cc. of Undiluted Washed Sheep Cells and Tested 5, 8, 11 and 14 Days after Injection.

Rat No.	Operation	Interval between operation and injection	Amount injected	Titer				Weight at operation	Weight at death	Weight of thymus
				5th day	8th day	11th day	14th day			
		days						gms.	gms.	mg.
115	Suprarenalectomy	14	1 cc. undiluted	6,000	1,000	Dead		250	195	313
116	"	"	"	16,000	3,000	1,800	200	250	210	—
117	"	"	"	8,000	4,000	2,000	200	200	180	214
118	"	"	"	8,000	3,000	Dead		230	215	516
119	"	"	"	2,000	800	400		265	210	375
121	"	"	"	4,000	3,000	1,000		270	250	320
122	Traumatization	"	"	8,000	2,000	800	160	270	300	210
123	"	"	"	18,000	4,000	Dead		168	160	155
124	"	"	"	6,000	3,000	160		225	220	410
139	Control	—	"	4,000	600	400	30			
140	"	—	"	4,000	2,000	800	80			
141	"	—	"	1,600	400	160				
142	"	—	"	1,600	1,600	800	200			
143	"	—	"	800	2,000				200	170
144	"	—	"	1,600	200	200			200	110
151	"	—	"	1,000	1,000	1,000	400			
152	"	—	"	1,600	1,600					
153	"	—	"	2,000	600	400	200		505	115

mized rats injected with 1 cc. undiluted sheep cells correspond to the titers obtained in normal rats injected with one-tenth this quantity, but exceed titers obtained in normal rats injected with the same quantity.

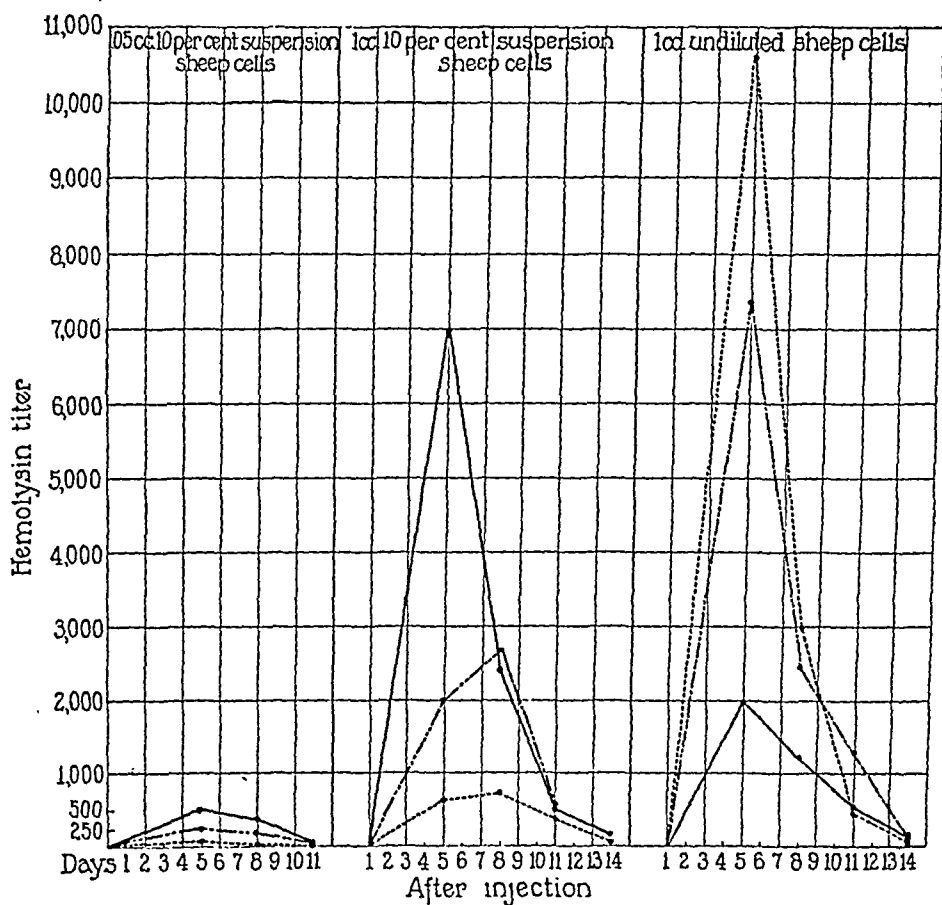


FIG. 2. Hemolysin formation with varying quantities of antigen in normal, suprarenalectomized and traumatized rats. The curves indicate the course of the average hemolysin formation following a single intraperitoneal injection of .05 cc. of a 10 per cent suspension, 1 cc. of a 10 per cent suspension and 1 cc. of undiluted sheep cells respectively, 2 weeks after the operation.

———— Normal rats.

----- Suprarenalectomized rats.

..... Traumatized rats.

Hemolysin Formation in Rats Receiving a Single Intraperitoneal Injection of 1 Cc. of a 10 Per Cent Suspension of Sheep Cells 2 Weeks after Traumatization of the Perisuprarenal Tissue.

Throughout the experiments reported in this paper, it had been observed that the traumatized control rats in all postoperative periods

TABLE VII.

Hemolysin Formation in Bilaterally Traumatized Rats, Intraperitoneally Injected at Varying Days after the Operation with 1 Cc. of 10 Per Cent Suspension of Washed Sheep Cells and Tested 5, 8, 11 and 14 Days after the Injection.

Rat No.	Operation	Interval between operation and injection	Amount injected	Titer				Weight at operation	Weight at death	Weight of thymus
				5th day	8th day	11th day	14th day			
		days						gms.	gms.	gms.
59	Traumatization	2	1 cc. 1/10	16,000	20,000	3,000		160	165	162
60	"	"	"	800	800	400	160	150	155	198
36	"	7	"	1,800	3,000	800	40	175	175	273
37	"	"	"	800	800	40	80	190	183	223
75	"	14	"	1,000	800	1,600	80	260	270	275
76	"	"	"	160	40	20	0	222	185	295
57	"	28	"	180	200	80		220	280	185
58	"	"	"	3,000	800	160		170	280	185
125	"	14	"	20	40	40	100	210	280	190
127	"	"	"	800	160	100	150	205	265	171
128	"	"	"	20	200	20	100	216	280	155
129	"	"	"	800	600	200		235	250	
130	"	"	"	400	800			210	280	140
131	"	"	"	800	1,600	200	100	165	185	75
132	"	"	"	300	1,600	400	100	190	210	80
133	"	"	"	2,000	1,600	400	100	155	195	95

including 4 weeks gave, with few exceptions, strikingly low titers. This finding was so striking that in a series of rats the perisuprarenal tissue was traumatized, and the rats were subsequently injected with 1 cc. of a 10 per cent suspension of sheep cells 2 weeks after the operation. The titers were tested on the 5th, 8th, 11th and 14th days after injection. Rats 125 to 133 tabulated in Table VII record the results

of this series. One of the nine rats gave a titer of 1:2000; all the others were below 1:800. These titers corresponded in general with those given by the suprarenalectomized rats during the 1st week. This profound depression was still observed in Rat 57, 4 weeks after the operation.

In four rats, one kidney was removed as an operative control. These rats were injected 2 weeks after the operation with 1 cc. of a 10 per cent suspension of sheep cells. As indicated in Table VIII the titers were the same as in the normal unoperated rats.

TABLE VIII.

Hemolysin Formation in Unilaterally Nephrectomized Rats, Intraperitoneally Injected 14 Days after Operation with 1 Cc. of 10 Per Cent Suspension of Washed Sheep Cells and Tested 5, 8, 11 and 14 Days after the Injection.

Rat No.	Operation	Interval between operation and injection	Amount injected	Titer				Weight at operation	Weight at death	Weight of thymus
				5th day	8th day	11th day	14th day			
		days						gm.	gm.	mg.
99	R. removed	14	1 cc. 1/10	8,000	400	400		140	145	110
100	"	"	"	12,000	200	600		145	145	233
101	"	"	"	1,600	200	200	80	230	235	230
102	"	"	"	32,000				200	205	209

It is justifiable to conclude from these observations that traumatization of the perisuprarenal tissue in some way results in a prolonged depression in antibody-forming capacity. This depression persists at the same low level from a few days after the operation to at least the 5th week. Traumatization of the perisuprarenal tissue in rats results in a depression of hemolysin formation which is even more marked than in a corresponding suprarenalectomized series at the same postoperative interval. It is suggested that traumatization of the perisuprarenal area probably results in severing or injuring the sympathetic or parasympathetic nerves innervating the suprarenals. This injury may cause a depression of function of cortex as well as medulla.

It has been thoroughly established by the work of Dreyer (11), Tscheboksaroff (12), Stewart and Rogoff (13) and others that the output of epinephrine from the suprarenal gland is controlled by a nervous mechanism and that interruption of the nervous pathway results in a diminution of the epinephrine output. The extent of the nervous control of the cortex, however, is entirely unknown. Depression of antibody-forming capacity to small amounts of antigen may be an expression of impaired suprarenal function. With the removal of suprarenal glands in rats, regeneration of thymus and lymphoid tissue occurs. This hyperplasia does not occur following traumatization of perisuprarenal tissue. This lymphoid hyperplasia may be in some way responsible for the partial recovery in suprarenalectomized rats of the antibody-forming capacity 2 weeks after the operation.

DISCUSSION.

It has been thoroughly established that the resistance of suprarenalectomized rats and rabbits to injections of various toxins is markedly diminished particularly during the first few weeks following the operation. As has been pointed out by Marine (14) and others, it is not established that antibody formation is necessarily an indication of resistance. However, it would appear *a priori* that during the height of diminished resistance, some depression in antibody reaction capacity may be expected. Our studies indicate that certainly in the 1st week following suprarenalectomy, a marked hemolysin depression does occur and is most marked at the height of susceptibility. The significant chemical changes in the blood plasma known to follow suprarenalectomy may play a rôle in this depression. Physicochemical changes such as change in the dispersion of the proteins in the serum, slight variations in the pH, variations in the concentration of metallic ions in the serum may all play a part in the depression in hemolysin during the early period following suprarenalectomy. However, the serum of suprarenalectomized rats when added in varying concentrations to sera of known hemolytic titer did not produce any depression of hemolysis. This adds further evidence that the depression in antibody formation in the early period after suprarenalectomy may be due to a disturbance in the antibody-forming mechanism of the body. This depression coincides with the di-

minished resistance of rats to toxins and poisons during the first 2 weeks and may be intimately related to it.

Marine and his coworkers (1, 4) found an increase in antibody formation following suprarenalectomy. They employed sublethal amounts of antigen. Their results are comparable with those in our work in which large amounts of antigen were used. Periods earlier than 3 weeks were not tested by these investigators. The effects of comparably small amounts of sheep cells were not tried. The rise in antibody production following the use of large amounts of sheep cells 2 weeks after suprarenalectomy may be indirectly dependent on loss of suprarenal function and on the consequent lymphoid hyperplasia following suprarenalectomy.

Traumatization of the perisuprarenal tissue had an effect similar to that of suprarenalectomy in depressing hemolysin formation particularly during the 1st week following the operation. This depression persisted in all periods up to 4 weeks. It is interesting to note, however, that with ten times the normal optimum antigen amount, the titers in the traumatized as well as in the suprarenalectomized rats were higher than in normal rats.

SUMMARY.

In a large series of suprarenalectomized rats, hemolysin formation, to a fixed amount of sheep cells injected intraperitoneally (1 cc. of a 10 per cent suspension), was studied 48 hours, 7, 14 and 28 days after operation. The hemolysin formation was studied in suprarenalectomized rats injected 2 weeks following the operation, with one-twentieth and ten times this amount. Hemolysin formation in rats traumatized by tearing the perisuprarenal tissue and injected 2 weeks after the operation with 1 cc. of a 10 per cent suspension of sheep cells was studied and the results compared with those in the normal control rats. The effect of the operative procedure was controlled by removing one kidney in a series of rats and determining the antibody response to sheep cells.

1. Bilateral suprarenalectomy in rats subsequently injected intraperitoneally with 1 cc. of a 10 per cent suspension of sheep cells resulted in a depression of hemolysin titer during 5 weeks following the operation, the depression being most marked during the 1st week.

2. Bilaterally suprarenalectomized rats injected intraperitoneally 2 weeks after operation with 1 cc. of undiluted sheep cells gave hemolysin titers higher than did normal rats.

3. The quantity of antigen necessary to yield the maximum titer in suprarenalectomized rats 2 weeks after operation is ten times the quantity necessary to yield the same titer in normal rats.

4. Traumatization of the perisuprarenal tissue in rats produced the same effect on the antibody-forming capacity as suprarenalectomy.

We wish to thank Dr. David Marine for his helpful advice and criticism throughout the course of this work.

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AN EXPERIMENTAL STUDY OF DIATHERMY.

V. THE ELEVATION OF TEMPERATURE IN THE PNEUMONIC LUNG.

By RONALD V. CHRISTIE, M.B., CH. B., WILHELM EHRLICH, M.D.,
AND CARL A. L. BINGER, M.D.

(From the Hospital of The Rockefeller Institute for Medical Research.)

PLATES 34 AND 35.

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INTRODUCTION.

Two of us (C. B. and R. C.) have shown that the systemic temperature can be raised by diathermy, but that there is relatively little local heat developed in the normal lung (1). Thermocouples placed in the lobes of the lungs of anesthetized dogs seldom registered more than 0.4°C . higher than simultaneously recorded rectal temperatures. This is true in spite of the fact, now established (2), that the high frequency current produced by a diathermy machine actually penetrates the body and passes through the lungs, generating heat in the tissues it traverses. Why there is no marked local elevation of temperature in these tissues has been shown to be due to the fact that the heat generated is rapidly disseminated throughout the body by the circulating blood. The blood leaving the lungs can be shown to have been heated by the current (3). If, however, the pulmonary circulation to one lung is interrupted, a precipitous rise in temperature occurs in the ischemic lung (4). The degree of local heating which occurs under these circumstances will depend upon the extent of interference with the pulmonary circulation. This can be shown by interrupting the flow of blood through one branch of the pulmonary artery, leaving the veins patent and the bronchial circulation intact. There results from this procedure an increase of temperature in the ligated lung amounting to about 1.5°C . in excess of the temperature in the normal lung. The increase occurs immediately after the artery is clamped, while the subsequent rate of heating is similar to that in the control

lung. When, on the other hand, the veins to one lung are ligated, the local heating is far greater than under the conditions just described. Reference to the figures in Paper II of this series will bring out these facts.

From the foregoing, the implication seems obvious that a disease process accompanied by impairment of local circulation may be expected to provide conditions consistent with the production of local heat by the passage of high frequency currents through the region involved. That the pulmonary consolidation of pneumonia represents a disease process in which local circulatory impairment exists, there is evidence to believe. We cite in this connection the pathological studies of Kline and Winternitz (5), the injection preparations of Gross (6) and the inferences to be derived from analyses of the oxygen saturation of the arterial blood (7). The present study was undertaken for the purpose of discovering whether the consolidated lobe could in fact be heated above the temperature of the uninvolved, relatively normal lung tissue. The difficulties attending the production of experimental lobar pneumonia in laboratory animals are well known. Though the pathological lesions which presented themselves in the experiments about to be reported were not perhaps identical with those commonly seen in human lobar pneumonia, they, at least, may be said to simulate this condition and to represent a more or less complete consolidation of a lobe, or the major part of it.

We have found that such a consolidated lobe can be heated by a diathermy current of the strength generally used in therapy to a point approximately 1°C. to 2°C. above the temperature of the surrounding normal lobes.

Methods.

Dogs were used in all of these experiments. During deep ether anesthesia they were inoculated by the method of intrabronchial insufflation described by Lamar and Meltzer (8). Actively growing broth cultures of two varieties of organisms were used: *B. friedländeri* Type B and *Pneumococcus* Type I. An effort to maintain virulence was made in the case of Friedländer's bacillus by passage through guinea pigs, and in the case of pneumococcus by mouse passage. The cultures were injected intrabronchially in volumes varying from 1 to 3 cc. per kilo body weight. Even with careful control of conditions the results of these inoculations were uncertain and variable. Some animals died within 24 hours of inoculation, before the appearance of a pulmonary lesion. Others survived ap-

parently unharmed. It was necessary to select animals for use on the basis of x-ray evidence of pneumonic consolidation.

The method of lung temperature measurement and diathermy administration was the same as that previously described (1). Observations were made on the dogs after they had been anesthetized by the intravenous injection of a 10 per cent solution of barbital-sodium, the dose not exceeding 0.3 gm. per kilo body weight. The low initial temperatures often observed may be ascribed in part to the result of infection, in part to heat loss resulting from anesthesia. This was difficult to prevent, since we thought it best to use no hot pad or source of heat other than diathermy. Considerable practice was necessary before we learned to lodge the thermocouple needle in the substance of the consolidated lobe. Owing to the differences in the shapes and sizes of the dogs it was difficult to find any external landmarks which would act as guides for the insertion of the needle into any particular area. In the majority of instances the lower lobe alone was consolidated. In order to get a thermocouple into it the needle was usually inserted close to the spinal column, through the 9th or 10th intercostal space. Even with this guide the element of luck remained a large one. Other thermocouples were inserted in symmetrical positions into the opposite healthy lobes. At the close of each experiment an autopsy was done in which the positions of all thermocouples were located. The gross appearance of the lungs was described in relation to the site of the thermocouples and the lungs were usually saved either for injection preparations or for microscopic examination.

EXPERIMENTAL.

Experiment D 36.—May 19, 1927. At 2:45 p.m. a male mongrel fox terrier weighing 12.7 kilos was given $\frac{1}{2}$ gr. morphine sulfate subcutaneously. About 15 minutes later it was etherized deeply. A short piece of rubber tubing was passed through the larynx into the trachea, and through this was inserted a soft rubber tube 30 cm. long, with an outside diameter of 3 mm. When the bronchial catheter was in place its proximal end was connected with the nozzle of a record syringe previously filled with 26 cc. of a 4 hour broth culture of *B. friedländeri*. The whole mass of culture was then injected and forced into the lung with a small quantity of air. There was no cough following injection. The animal made a quick recovery from the anesthetic.

May 20, 12, noon. The dog looked moderately sick. The respirations were rapid, but not labored. Rectal temperature was 39.5°C.

May 21, 9 a.m. The dog was now definitely sick. An x-ray photograph showed a slight shadow at the base of the left lung (Fig. 1).

May 21, 10 a.m. The animal was anesthetized by the intravenous injection of 27 cc. of a 10 per cent solution of barbital-sodium.

10:30 a.m. The sides of the dog's thorax were shaved and lead-tin electrodes 3 × 4 inches square were applied to the shaved skin. To assure perfect contact several layers of gauze moistened with a solution made from mixing equal parts of

glycerol and saturated saline were interposed between skin and electrodes. A rectal thermometer was put in place and the thermocouples were thrust through the chest wall into the lungs.

11:10 a.m. The larynx was intubated to facilitate breathing.

11:14 a.m. to 1:50 p.m. Thermocouple readings were made at 10 to 15 minute intervals (Table I). The diathermy current was turned on at 11:29 a.m. and ran continuously until 1:50 p.m.

1:55 p.m. The dog was killed by the intravenous injection of 20 cc. of a saturated solution of $MgSO_4$, the trachea being clamped in inspiration.

The thorax was opened and the position of the thermocouples and condition of the lungs established.

Thermocouple 1 was buried in the substance of the lower portion of the left upper lobe, near the lung root. It was lying in a discolored, congested area of the lobe which was, however, air-containing.

Thermocouple 3 had transfixed the upper pole of the left lower lobe and lay in that portion of the ventral lobe which adjoins the hilum. This part of the lobe was dark and definitely abnormal in appearance, as was the left lower lobe immediately adjoining it, which was purplish, boggy and non-air-containing. No thermocouple was found in this left lower lobe.

Thermocouple 2 was found in the substance of the right upper lobe in what was apparently normal lung tissue.

Thermocouple 4 lay in the pleural cavity projecting into the interlobar space and surrounded by healthy tissue.

To summarize the situation found at autopsy: Two of the thermocouples, Nos. 1 and 3, were found within the substance of the left lung, which was the seat of a pneumonic process. Although lying in pathological tissue, neither had penetrated the lower lobe, where the consolidation was most pronounced. The other two thermocouples, Nos. 2 and 4, lay in, or in close proximity to, the normal tissue of the right lung.

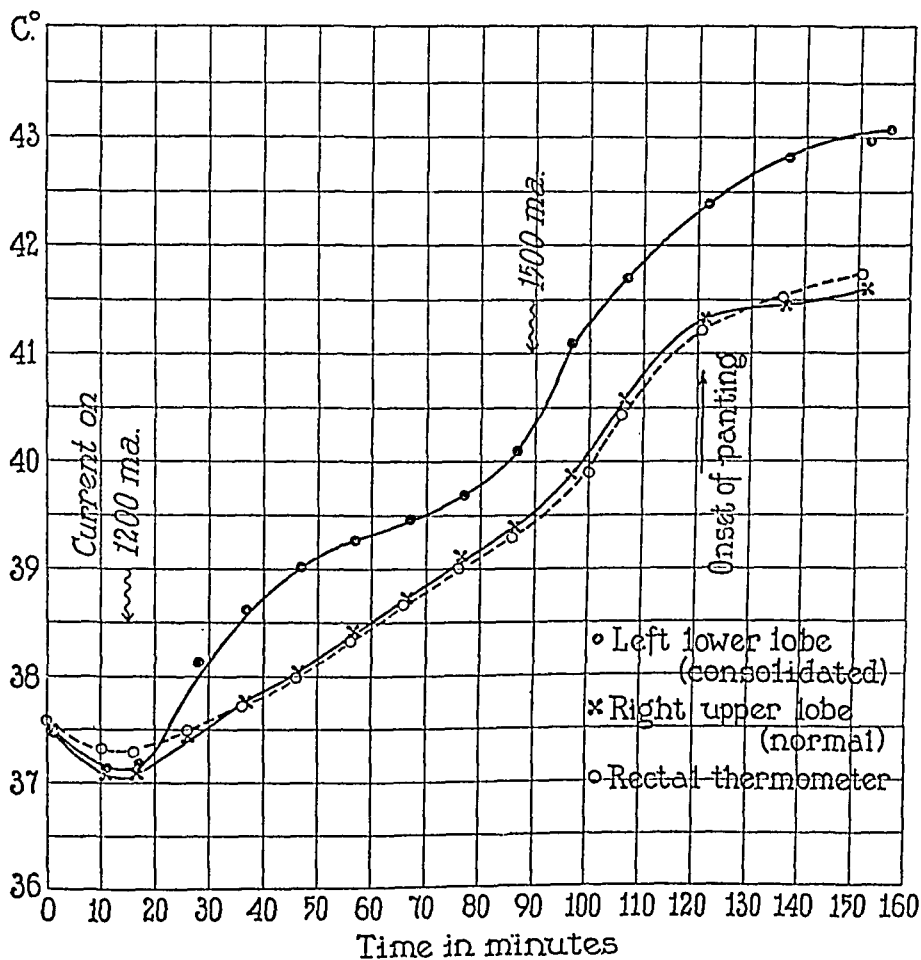
As to the temperature changes recorded by these thermocouples: Both thermocouples, Nos. 1 and 3, showed throughout the duration of current flow a greater rate of heating than Nos. 2 and 4, which were lodged in the opposite normal lung. The rate was greater in No. 3 than in No. 1, corresponding in this to the degree of structural damage found at the respective sites of these thermocouples. Comparing the temperatures developed in the left lung and right lung (Table I, Text-fig. 1) one sees, for example, in Thermocouple 2 (right healthy lung) a rise of $4.52^{\circ}C.$ in 2 hours and 21 minutes of diathermy. The rectal temperature during this period had risen $4.42^{\circ}C.$, while Thermo-

TABLE I.
Temperature Changes Recorded in Experiment D 36.

Time	Current ma.	Rectal thermom- eter °C.	Thermocouple 1 in left lung (pathologic)	Thermocouple 2 in right lung (normal)	Thermocouple 3 in left lung (pathologic)	Thermocouple 4 in right lung (normal)	Thermocouple 5 in subcutaneous tissue under left electrode	Thermocouple 6 on skin under left electrode	Thermocouple 7 on skin under right electrode	Pulse rate per min.	Respiratory rate per min.	Remarks
11:14	0	37.57*	37.50	37.48	37.48	37.51	35.98	35.17	36.07	29	166	Respirations irregular
11:24	0	37.31	37.19	37.08	37.14	37.16	35.98	35.07	36.00	37	168	Current on at 11:29
11:30	1200	37.28	37.33	37.09	37.68	37.20	47.78	38.12	39.15	38	168	
11:40	1200	37.48	37.62	37.42	38.13	37.43	50.13	41.40	41.30	28	164	
11:50	1200	37.71	37.94	37.75	38.62	37.74	52.11	43.31	42.71	31	166	
12:00	1200	37.98	38.26	38.01	39.01	38.05	51.87	43.88	42.86	30	164	
12:10	1200	38.32	38.68	38.41	39.26	38.43	50.68	42.85	42.48	24	170	
12:20	1200	38.66	38.92	38.72	39.46	38.75	50.77	42.88	42.45	23	172	
12:30	1200	39.00	39.18	39.12	39.69	39.16	50.52	42.98	42.45	23	180	Current increased to 1500 ma. at 12:43}
12:40	1500	39.29	39.52	39.39	40.10	39.41	50.49	43.16	42.55	23	184	Rectal temperature read at 12:54
12:50	1500	39.89	40.14	39.88	41.09	39.99	56.30	45.29	44.38	25	186	Panting began suddenly at 1:16
1:00	1500	40.43	40.84	40.57	41.69	40.68	55.72	45.92	44.81	24	188	Panting.
1:15	1500	41.21	41.70	41.32	42.38	41.46	55.00	45.92	45.05		190	Panting. Current off at 1:50½
1:30	1500	41.52	42.07	41.45	42.82	41.60	54.65	45.88	45.32		194	
1:45	1500	41.73	42.25	41.60	42.98	41.75	54.12	45.88	45.29		204	
1:50	0				43.08							
1:50½	0				42.51							
1:50½	0				42.32							

*The rectal thermometer and Thermocouple 1 were read at the times indicated. Other readings were made at ½ minute intervals.

couple 3, in diseased lung, showed an increase of $5.84^{\circ}\text{C}.$, or $1.32^{\circ}\text{C}.$ above the temperature developed in the control lung. Such an increase corresponds in order of magnitude to the change shown to occur



TEXT-FIG. 1. Experiment D 36. Curve showing relative rates of heating in consolidated and normal lung tissue as compared with the rectal temperature. After 107 minutes of current flow the dog suddenly began to pant. This resulted in cooling of the normal lung, which thereupon fell below the rectal temperature. No such change occurred in the pathological lobe.

during diathermy in a lung after clamping its pulmonary artery (4). The experiment just described (D 36) was repeated in six other dogs. Because of the inconstant pathological changes produced by Fried-

länder bacillus insufflations, and because of the inherent uncertainty of directing the thermocouple needles into both consolidated and healthy lung tissue, it was impossible to demonstrate heating of the pathological lobe in all experiments. We have deliberately chosen for publi-

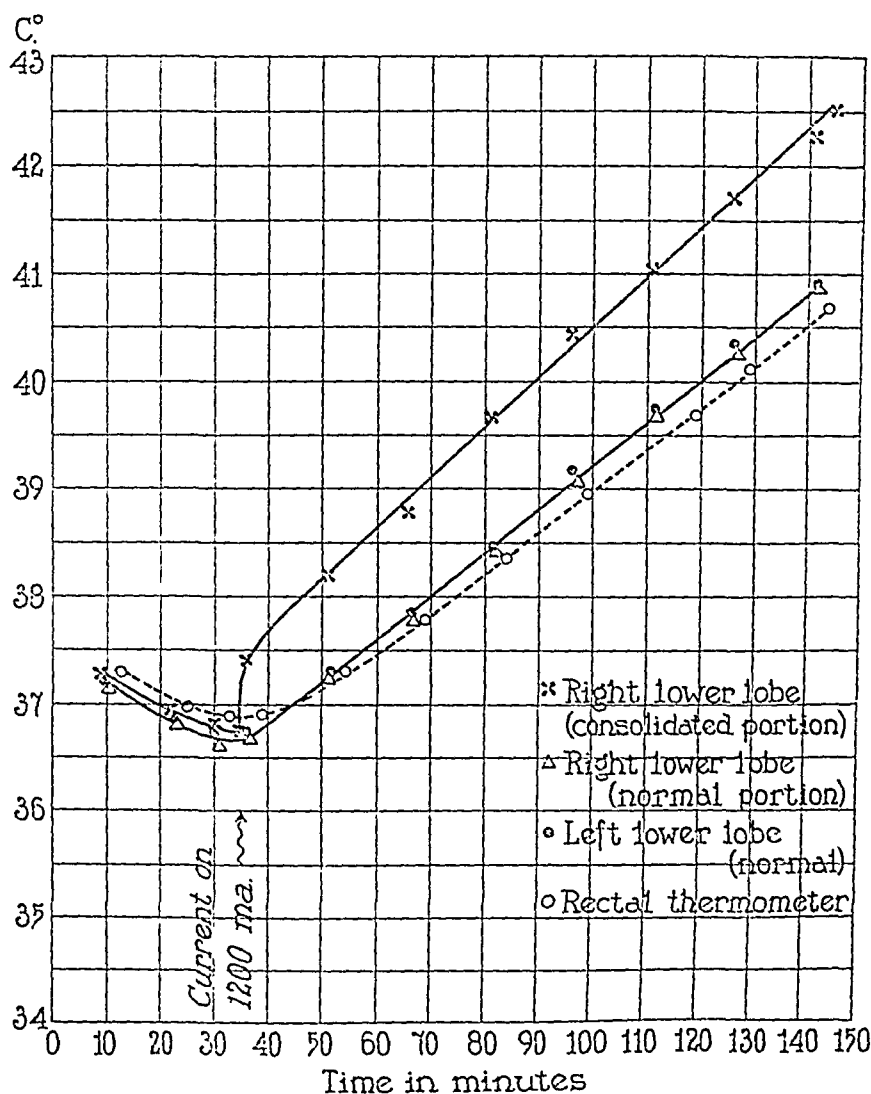
TABLE II.
Temperature Changes Recorded in Experiment D 38.

Time	Current	Rectal thermometer	Thermocouple 1 in right lung (consolidated)	Thermocouple 2 in left lung (normal)	Thermocouple 3 in right lung (pathological)	Thermocouple 4 in left lung (normal)	Thermocouple 5 in right lung (pathological)	Thermocouple 6 on skin under right electrode	Thermocouple 7 on skin under left electrode	Pulse rate per min.	Respiratory rate per min.
	ma.	°C.									
11:39	0	37.30*	37.29	37.20	37.18	37.18	37.29	34.52	34.76		48
11:52	0	36.98	36.94	36.86	36.82	36.84	36.92	34.60	34.76	184	52
12:00	0	36.89	36.82	36.73	36.62	36.68	36.77	34.80	34.80		64
	On										
12:05	1200		36.75								
12:06	1200	36.90	37.42	36.75	36.68	36.82	36.90	37.13	38.11	208	64
12:21	1200	37.31	38.20	37.31	37.25	37.44	37.46	40.78	41.02	200	62
12:36	1200	37.79	38.77	37.85	37.79	37.96	38.07	42.90	42.68	200	56
12:51	1200	38.37	39.67	38.49	38.44	38.60	38.70	43.59	43.14	200	50
1:06	1200	38.96	40.43	39.18	39.07	39.28	39.35	43.71	43.38	196	46
1:21	1200	39.07	41.01	39.77	39.67	39.90	39.98	43.75	43.83	196	44
1:36	1200	40.12	41.70	40.37	40.27	40.50	40.61	44.06	44.29	196	46
1:51	1200	40.69	42.25	40.90	40.86	41.10	41.26	44.73	44.81		
	Off										
1:55			42.51								
1:55½	0		41.89								
1:55¾	0		41.77								
1:55½	0		41.70								
1:56¾	0			41.02							

* The rectal thermometer and Thermocouple 1 were read at the times indicated. Other readings were made at ½ minute intervals.

cation in detail those experiments in which a certain degree of local heating was produced. It should be emphasized that failure to find local heating could always be accounted for on the basis of no consolidation or malposition of thermocouples, except in one instance, Experiment D 49, to be referred to below.

Experiment D 38.—On May 25, 1927, at 2:45 p.m., a male mongrel weighing 12.6 kilos, after a preliminary subcutaneous injection of $\frac{1}{2}$ gr. morphine sulfate, was given, under deep ether anesthesia, an intrabronchial insufflation of 25 cc.



TEXT-FIG. 2. Experiment D 38. Curve showing relative rates of heating in consolidated and normal lung tissue as compared with the rectal temperature.

of a $4\frac{1}{2}$ hour culture of *B. friedländeri*. The next afternoon at 4:20 the dog looked moderately sick and showed a rectal temperature of 40.1°C. An x-ray photograph showed a slight shadow at the base of the right lung (Fig. 2). On

May 27, at 9:30 a.m., the dog was coughing occasionally, but looked less sick than on the previous day. The animal received an intravenous injection of 37 cc. of a 10 per cent solution of barbital-sodium. When a satisfactory state of anesthesia had been achieved, electrodes were applied and thermocouples inserted in the usual manner. A current of 1200 milliamperes was passed through the thorax for 1 hour and 50 minutes. Immediately after the current was turned on it was found that Thermocouple 1 which had been directed into the right lower lobe through the anterior chest wall was recording a more rapid rise in temperature than any of the others within the thorax or than the rectal thermometer. This thermocouple, No. 1, was found at autopsy to be buried in the right lower lobe which had the appearance of liver, except for an air-containing portion on its anterior aspect. Both of the other thermocouples in the right lung were found in the upper portion of the lower lobe where the consolidation was less marked. The lesion on the right side increased in severity from above downward, which may perhaps account for the fact that the final temperatures observed in the right lower lobe showed a gradation from above downward: 40.86°C., 41.26°C., 42.25°C. The final temperature recorded by this thermocouple represented a rise of 5.45°C. in 1 hour and 50 minutes (Table II, Text-fig. 2), as compared with a rise of 4.17°C. and 4.42°C. respectively in thermocouples, Nos. 2 and 4, located in the normal left lower lobe. The increase in rectal temperature during the same interval amounted to 3.80°C. The final temperature of the skin under the electrodes was 44.73°C. on the right side, 44.81°C. on the left. The surface temperature cannot, therefore, be held responsible for the observed difference in internal temperature.

The findings in Experiment D 38 are quite comparable to those in D 36. In interpreting them the many controls supplied by our previous studies should be borne in mind, where it was clearly shown that the normal lung with intact circulation could be heated to an average maximum of 0.35°C. above the rectal temperature. In only one experiment of 13 did the temperature in the lung mount as high as 0.6°C. above the rectal during the course of diathermy. Since this animal died unaccountably while the experiment was in progress it is probable that the slight increase in heating can be explained on the basis of a failing pulmonary circulation.

Experiment D 48.—The procedure outlined in the previous experiments was again carried out in a female mongrel weighing 8.8 kilos. While etherized it received, on Nov. 6, 1927, 10 cc. of a *B. friedländeri* culture intrabronchially. The next afternoon lung temperature measurements were recorded during diathermy. Autopsy showed the left lower lobe to be hepatized, while the upper and ventral lobes were air-containing and normal in appearance. The right lung was normal except for congestion and nodular consolidations along the posterior

margin of the lower lobe, most marked near the hilum. The caudal lobe, as well, was congested. The most marked temperature increase was recorded by one of the two thermocouples in the left lower hepatized lobe which showed a final rise of $1.62^{\circ}\text{C}.$ above the rectal temperature. A second thermocouple in this lobe showed an increase of only $0.6^{\circ}\text{C}.$ above the rectal, similar to the change observed in the thermocouple in the opposite lobe.

In this experiment a careful histological examination of the lungs was made particularly with an eye to finding evidence of circulatory obstruction. Such evidence was indeed found. The microscopic examination was made independently by one of us (W. E.) who was not informed of the other experimental findings.

Result of Microscopic Examination of Lungs in Experiment D 48.—(Figs. 4 and 5.) The lungs were distended with Zenker's fluid and immersed in it. Sections were taken from all lobes and imbedded in paraffin. These were cut to a thickness of 5 to 6 micra and stained with eosin-methylene blue, iron-hematoxylin-eosin and Gram's stain. The findings are given below:

Right upper lobe: The section appears normal.

Right ventral lobe: The section appears normal.

Right lower lobe: One section appears normal. A second, however, at its center shows certain pathological changes. The bronchioles and alveoli are uniformly filled with an exudate consisting of alveolar epithelial cells and polymorphonuclear leucocytes. The epithelial cells are often filled with vacuoles and with Gram-negative bacilli, often also with polymorphonuclear leucocytes and erythrocytes. There is everywhere a proliferation of young epithelial cells from the alveolar walls and polymorphonuclear leucocytes are to be seen within the walls of the bronchioles and bronchi. The walls of the blood vessels are edematous in places. In the smaller vessels the adventitia frequently shows a proliferation of round cells. The capillaries are dilated with blood and the lymph vessels of the pleura are dilated with lymph. The exudate diminishes toward the peripheral portions of the section where the alveoli appear normal.

Left upper lobe: In the center of the section there is an area similar to the one just described, but smaller.

Left lower lobe: In three sections one sees the alveoli and bronchioles uniformly filled with exudate. There are no air-containing ones to be seen. The exudate has the same composition as described in the right lower lobe but is apparently greater in quantity. In some places where the exudate is plentiful there are comparatively few old, mostly phagocytosed erythrocytes. Here the capillaries are compressed and contain no red blood corpuscles (Fig. 5). Where there is less exudate, well preserved erythrocytes and some fibrin are often to be seen lying in the alveolar spaces. Here the capillaries are dilated with blood. The walls of the large vessels are edematous and infiltrated with many erythrocytes. The epithelial

lial lining of the bronchioli is mostly desquamated, that of the larger bronchi is well preserved. Many of the bronchial walls exhibit hemorrhages. The lymph vessels are dilated, containing erythrocytes, polymorphonuclear leucocytes and fibrin.

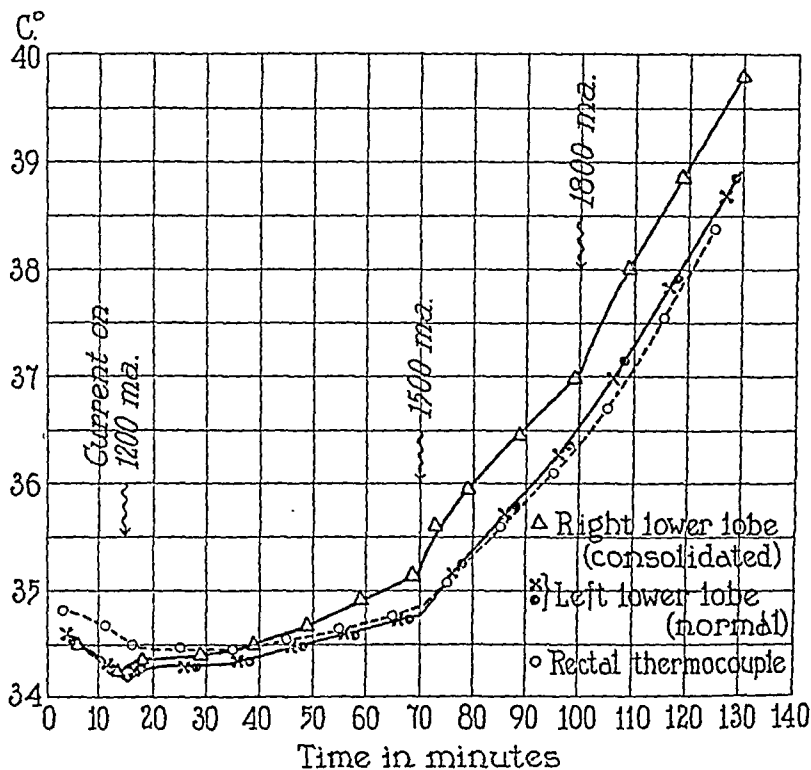
The significant finding is the compressed empty capillaries. Apparently this is due to the pressure of the exudate and depends for its occurrence upon the amount of exudate. This ischemic condition was found only in certain parts of the left lower lobe. One of the two thermocouples in this lobe showed a rise of temperature greater than that occurring in any of the other lobes. The second thermocouple, however, recorded an increase in temperature equal to that in the right lower lobe. The experimental findings are then quite consistent with the changes observed by microscopic study.

In Experiment D 49 the dog received 1 cc. per kilo of an actively growing culture of *B. friedländeri*. Observations were made the following day. The dorsal half of the right lower lobe was consolidated. There was no local heating recorded by either of the two thermocouples imbedded in the pathological lobe. The final temperatures recorded in the consolidated portion of the right lower lobe were 37.75°C. and 37.87°C. after 42 minutes of diathermy at 1200 milliamperes, as compared with a temperature of 37.97°C. in the relatively normal left lower lobe. The rectal temperature recorded simultaneously by a mercury thermometer was 37.68°C. In this animal the microscopic picture of the right lower lobe (Fig. 6) showed the alveoli to be very irregularly filled with exudate. Air-containing alveoli were seen lying between those showing the inflammatory reaction. The capillaries, far from being compressed and empty, were dilated with blood. A similar dilatation of vessels, but no exudate, was found in the left lower lobe. It seems not improbable that the lack of local heating was associated with the absence of vascular obliteration.

A number of experiments was made, using cultures of *Pneumococcus* Type I in place of *B. friedländeri* for intrabronchial insufflation. It was hoped that the fibrin reaction, usually stimulated by Type I pneumococcus, would cause a greater interference with the pulmonary circulation and therefore permit of more definite local heating. This was not the case. The response of dogs to intrabronchial insufflations of Type I pneumococci is an extremely variable one. They will often

tolerate large amounts of actively growing mouse-virulent cultures when injected by this route. We have selected for publication one experiment (D 54) in which a pneumonic consolidation was produced involving the caudal lobe and three-quarters of the right lower lobe, where a thermocouple had been placed.

The cut section of the right lower lobe was solid and apparently non-air-containing. Histological examination of the right lower lobe showed the alveoli and bron-



TEXT-FIG. 3. Experiment D 54. Curve showing relative rates of heating in consolidated and normal lung tissue as compared with the rectal temperature.

chioles mostly filled with exudate. In some places air-containing alveoli occurred amongst the others. The exudate consisted chiefly of polymorphonuclear leucocytes mixed with varying numbers of vacuolated alveolar epithelial cells often containing erythrocytes and degenerated cocci. Most of the capillaries were dilated with blood. Red cells were seen in the alveolar exudate and in some places hemorrhages were present in the walls of the bigger vessels. No evidence of capillary fibrin thrombi was to be found. The left lung was apparently quite normal though sections from the lower lobe showed that the vessels were dilated

with blood. Difference in temperature between the pathological and normal lobes was slight, but became more apparent on increasing the milliamperage to 1800 (Text-fig. 3). The final temperature in the consolidated lobe was $1^{\circ}\text{C}.$ above the rectal temperature while the normal lung was $0.15^{\circ}\text{C}.$ above the rectal temperature. The slight degree of elevation of temperature in the involved lobe produced by the current of 1200 milliamperes may be explained on the basis of no clear evidence of a grossly impaired circulation, since neither capillary thrombi were present nor sufficient exudate to compress the capillaries. For the higher current strengths it is probable that the existing circulatory disorder was sufficient to permit of a moderate degree of local heating.

The existence of obstruction to the circulation was further indicated by injection preparations made with the technique described by Gross (6). Satisfactory preparations could not be obtained at each attempt. The final picture depended upon the conditions prevailing during injection. The presence of puncture holes caused by the thermocouple needles made it impossible to use the lungs, in most of these experiments, for purposes of injection.

We present in Fig. 3 the photograph of a cleared dog's lung injected immediately after death with a barium-gelatin mixture. 2 days before, the animal (D 35) had been injected intrabronchially with 25 cc. of a broth culture of *B. friedländeri*. It was killed by injecting 20 cc. of a saturated solution of MgSO_4 intravenously. The area of involvement described in the autopsy notes corresponds exactly to that portion of the lung which has injected poorly. We publish this photograph as further evidence for the impairment of the circulation in the pneumonic lung.

DISCUSSION.

We have deliberately chosen for publication only those experiments in which the proper combination of circumstances made it possible to demonstrate local heating of the lung by diathermy. These circumstances consist first of the development of a pulmonary lesion sufficient to cast a shadow when photographed by x-ray, and secondly of the accurate locating of thermocouples in both consolidated and normal lung tissue. When these conditions have been fulfilled we have been able to demonstrate local heating in three of four animals inoculated intrabronchially with cultures of *B. friedländeri* and in one animal inoculated in a similar fashion with a culture of *Pneumococcus*

Type I. The variation of heat production in these selected experiments can be accounted for by the positions of the thermocouples and by the degree of impairment of the pulmonary circulation. The pathological process in these dogs' lungs presents three stages: (1) a stage of active congestion (Fig. 6) in which relatively little exudate is to be seen and the alveolar capillaries are dilated with blood; (2) a transitional stage (Fig. 4) in which the amount of exudate is increased and the erythrocytes lying in the capillaries have lost their regular shapes and usual staining properties, and finally (3) an ischemic stage (Fig. 5) in which all the alveoli are uniformly filled with exudate and the capillaries are compressed and free from erythrocytes. This final bloodless stage in which the local circulation is definitely impaired is the one in which we believe a certain degree of local heating with diathermy may occur.

We have purposely drawn no inferences as to heat production during diathermy in the lung of a patient suffering from pneumonia.

SUMMARY AND CONCLUSIONS.

1. An experimental pneumonia with more or less lobar distribution has been produced in dogs by the method of intrabronchial insufflation of *B. friedländeri*, Type B, and *Pneumococcus*, Type I.

2. Such dogs as showed evidences of a pulmonary lesion when photographed by x-ray were selected for lung temperature measurements.

3. Measurements of lung temperature were made by means of thermocouples before and during diathermy.

4. The thermocouples which recorded the temperature in the consolidated lobes showed in most instances a more rapid rate of heating during diathermy than those in the normal lobes. The final increase in temperature in the pathological lobes over the normal lobes amounted to slightly more than 1°C.

5. When local heating occurred during diathermy it was of the order of magnitude found in a lung in which the branch of the pulmonary artery supplying it had been clamped.

6. Histological examination of the lungs showed the pathological reaction to consist of intraalveolar exudate composed of polymorphonuclear leucocytes and desquamated alveolar epithelium. In some

sections the exudate was sufficient to cause compression and emptying of the alveolar capillaries.

7. The local heating, we believe, depends upon this ischemic state of the smaller vessels.

8. Further evidence for an impaired circulation in the pneumonic lung is furnished by injection preparations in which the uninjected area corresponded exactly to the gross pathological lesion.

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EXPLANATION OF PLATES.

PLATE 34.

FIG. 1. X-ray photograph of thorax of Dog D 36 taken 42 hours after intra-bronchial insufflation with culture of *B. friedländeri*. The picture shows a slight shadow at the base of the left lung.

FIG. 2. X-ray photograph of thorax of Dog D 38, taken 26 hours after intra-bronchial insufflation with culture of *B. friedländeri*. The picture shows a slight shadow at the base of the right lung.

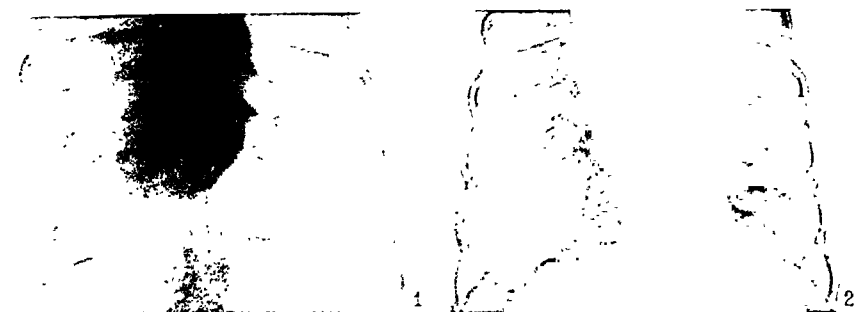
FIG. 3. Experiment D 35. Photograph of cleared barium-gelatin injection preparation of dog's lung in which consolidation had been produced by the intra-bronchial insufflation of a culture of *B. friedländeri*. The uninjected area corresponded to the part of the lung which showed gross pathological changes at autopsy.

PLATE 35.

FIG. 4. Experiment D 48. Left lower lobe. Magnification $\times 400$. The photograph shows the transitional stage in which the erythrocytes in the capillaries have become irregular in shape and have lost their usual staining properties. Methylene blue and eosin.

FIG. 5. Experiment D 48. Left lower lobe. Magnification $\times 400$. This represents the final ischemic stage. All the pulmonary alveoli are uniformly filled with exudate, the capillaries being compressed and bloodless. Methylene blue and eosin.

FIG. 6. Experiment D 49. Right lower lobe. Magnification $\times 400$. The photograph represents the stage of active congestion in which the alveolar capillaries are dilated with blood. Methylene blue and eosin.





ON INDIVIDUAL DIFFERENCES IN HUMAN BLOOD.*

By K. LANDSTEINER, M.D., AND PHILIP LEVINE, M.D. :

(From the Laboratories of The Rockefeller Institute for Medical Research.)

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The existence of individual differences in human blood was shown long ago by means of normal isoagglutinins (1). Soon thereafter variations were found also in the blood of animals (goats) through the use of immune sera prepared by injecting goats with blood of different individuals of the same species (2). These observations were later extended to other animals.

Curiously enough the reactions with normal human isoagglutinins do not occur in a, so to speak, haphazard manner but they separate the human bloods into four sharply defined groups designated as O, A, B, and AB. (1, 3).** It seems superfluous to describe the well known properties of these four groups.

In contradistinction to the simple scheme encountered with human blood, are the findings of Todd and White (5) who studied the serum of cattle immunized against cattle plague with the blood of infected animals. Taking advantage of the isohemolytic properties of such sera they discovered a remarkable variety in cattle blood; and similar conditions have been observed in the blood of chickens (Landsteiner and Miller (6)).

Attempts have been made also to discover further differences in human blood in addition to the group distinction. Evidence along this line has been furnished by von Dungern and Hirschfeld (7) in experiments with absorbed normal animal sera. It is difficult however to apply this method to a systematic study and the work was not carried on further. Following the technique of von Dungern and Hirschfeld we found some differences (see also Landsteiner and

* See the preliminary reports in *Proc. Soc. Exp. Biol. and Med.*, 1927, xxiv, 600, 941.

**In the present paper the nomenclature adopted by the American Association of Immunologists is used (4).

Witt (8)) aside from the conspicuous ones characterizing the groups, or those ascribable to the subdivision of group A, or due to the agglutinable factor found by Schiff (9) in blood cells of group O (*cf.* Witebsky and Okabe (10), Hirszfeld (11)). Our experiments were not regularly successful and frequently variations of smaller order could not be confirmed on repetition of the tests.

Another way of showing distinctions among individual bloods but also somewhat difficult of application, was found in the reactions of "cold agglutinins" of normal human sera (12-14).

The present paper concerns itself with a method of differentiating human bloods which yielded clear-cut and reliable results. It is based upon the use of immune agglutinins.

Using this technique Hooker and Anderson (15) found that immune sera produced in rabbits by injection of blood of group O still contained agglutinins for O blood after absorption with cells of any other group. The authors were inclined to explain this effect on the assumption of a property common to group O bloods.

EXPERIMENTAL.

Our first observations were made in experiments with stock anti-human blood immune sera from rabbits. Out of forty-one sera four were found that, after exhaustion with one sample of human blood, still contained agglutinins acting on a majority of bloods of all four groups while other bloods were not agglutinated. These tests showed the existence of an agglutinable property unrelated to the isoagglutinogens A and B, and differing from the latter in that there was not found a corresponding isoagglutinin in human serum. Naturally endeavors were made to produce immune sera endowed with the peculiar property described, by injecting rabbits with bloods possessing the new quality which may be designated as M. This was found to be rather difficult because only a few of the rabbits produce potent sera specific for M. However, on immunizing a sufficient number of animals, several such sera were obtained.

Some of the immune sera exhibited a different effect. When they were absorbed with blood of the type M+, the supernatant fluid reacted intensely and selectively on certain blood specimens, thus revealing a second agglutinable property (N).

The production of antibodies for N by injecting positively reacting bloods succeeded easily and some such sera were found among our supply of anti-human blood immune sera.

As was to be expected, anti-N agglutinins were not found in normal human sera. Also in normal animal sera we have not yet detected agglutinins for M or N.

An immune serum for a third agglutinable factor P was prepared by injecting blood (of colored individuals) selectively acted upon by absorbed normal rabbit and beef serum (according to the method of von Dungern and Hirschfeld).

In order to prepare specifically reacting agglutinin solutions, the inactivated immune sera in a dilution of 1:15 to 1:30 were treated with half the volume of packed, washed blood cells lacking the respective agglutinogens. A second treatment with the same or a smaller quantity of blood was required ordinarily to remove completely the agglutinins acting on human blood in general. The mixtures were allowed to stand for 1 hour at room temperature and were centrifuged. The fluids for N were prepared at first in this manner; subsequently, as will be explained below, the mixture of blood and serum was kept for $\frac{1}{2}$ to 1 hour at 37–40°C.

The details of the procedure have to be determined in preliminary experiments and the absorbing blood must be selected with regard to the properties of the serum, *e.g.*, the presence of group agglutinins. Before setting up the main experiments the fluids were controlled by testing them with known bloods.

The tests were made by adding to 3 or more drops of the agglutinating fluids 1 drop of 2.5 per cent suspension of washed blood. The readings were made after the tests had stood for 2 hours at room temperature, or 1 hour at 37°C., if the fluids had been prepared at this temperature. The strength of the reaction is indicated as follows: F. tr. = faint trace; tr. = trace; $\frac{1}{2}$, +, $+\frac{1}{2}$, etc. + signifies clumps visible without magnification or with a hand lens (magnification 6X) or clumps of medium size seen in the microscopic field (magnification 100X); ++ signifies large clumps seen with the naked eye and +++ complete agglutination.

For the production of immune sera freshly drawn and citrated blood (mostly of group O), after washing, was injected into rabbits at weekly intervals. The first injection of 3 cc. was given intravenously; the following injections of 4 cc. each, intraperitoneally. The sera were tested by absorption 6 days after the third and each subsequent injection. The animals were bled (mostly after four or five injections) the day following the tests when the sera had a sufficient content of the desired antibodies, *i.e.*, when they gave powerful specific reactions after absorption. For the preparation of anti-M immune sera it seems preferable to inject bloods of the M+ N- type.

TABLE I.

The immune sera diluted 1:20 were absorbed with suitable blood lacking the particular agglutinin, as described. The N agglutinins were prepared by absorption at room temperature (see page 769). Readings were made after 2 hours at room temperature.

Blood No.	815	816	817	819	821	822	823	824	825	826	828	829	830	831	832	833	834	835	836	837	838	839	840	841
Group	O	O	O	B	O	O	B	O	B	O	O	B	O	A	O	A	O	O	O	O	A	O	A	O
Immune serum 1; agglutinins for M.....	++	+	++	++	++	0	++	0	++	++	++	0	0	++	++	++	++	++	++	++	++	++	++	0
Immune serum 18; agglutinin for N....	0	tr.	0	+	++	++	0	++	++	+	++	++	++	+	++	0	++	++	++	++	0	++	++	++
Immune serum 5; agglutinins for P.....	++	++	++	++	++	++	+	+	++	+	+	+	0	++	++	+	0	+	+	+	+	0	0	0

It was of considerable importance to have at one's disposal a number of individuals whose blood could be examined repeatedly. The work was facilitated also by keeping particular specimens in a mixture recommended by Rous and Turner (5 volumes of 5.4 per cent glucose solution, and 2 volumes of a 3.8 per cent sodium citrate solution, for 3 volumes of blood) (16). In this solution the erythrocytes were still agglutinable when the blood was kept sterile in the refrigerator for several weeks.

TABLE II.
Frequency of M in the Four Blood Groups.

Group ..		O		A		B		AB		Total Number	
Reactions for M		+	-	+	-	+	-	+	-	+	-
Men	Number .	299	64	285	48	114	21	34	15	732	148
	Percentage	82 4	17 6	85 6	14 4	84 4	15 6	69 4	30 6	83 2	16 8
Women	Number .	115	40	80	17	32	10	13	3	240	70
	Percentage	74 2	25 8	82 5	17 5	76 2	23 8	81 2	18 8	77 4	22 6
Total Number		414	104	365	65	146	31	47	18	972	218
Percentage .		79 9	20 1	84 9	15 1	82 5	17 5	72 3	27 7	81 7	18 3

TABLE III.
Frequency of N in the Four Blood Groups. Absorptions and Tests at 37°C.

Group	O		A		B		AB		Total No.	
Reactions for N	-	-	+	-	+	-	+	-	+	-
Total No	162	48	143	46	28	10	6	3	339	107
Percentage									76 0	24.0

A representative experiment on twenty-four blood samples taken at random with exhausted immune sera containing agglutinins for M, N, and P, respectively, is given in Table I.

The frequency (in white individuals) of the positive and negative reactions for the property M and their occurrence among the blood groups are presented in Table II. The distinction between positive and negative bloods for M was regularly sharp when the exhaustion was made with suitably selected bloods.

It is seen from the table that positive reactions are much more frequent than negative ones. The percentage figures for the four groups do not deviate greatly from the total average except for group AB, but here the number of individuals examined is too low to warrant any conclusion. A similar remark may apply to the figures for the sexes.

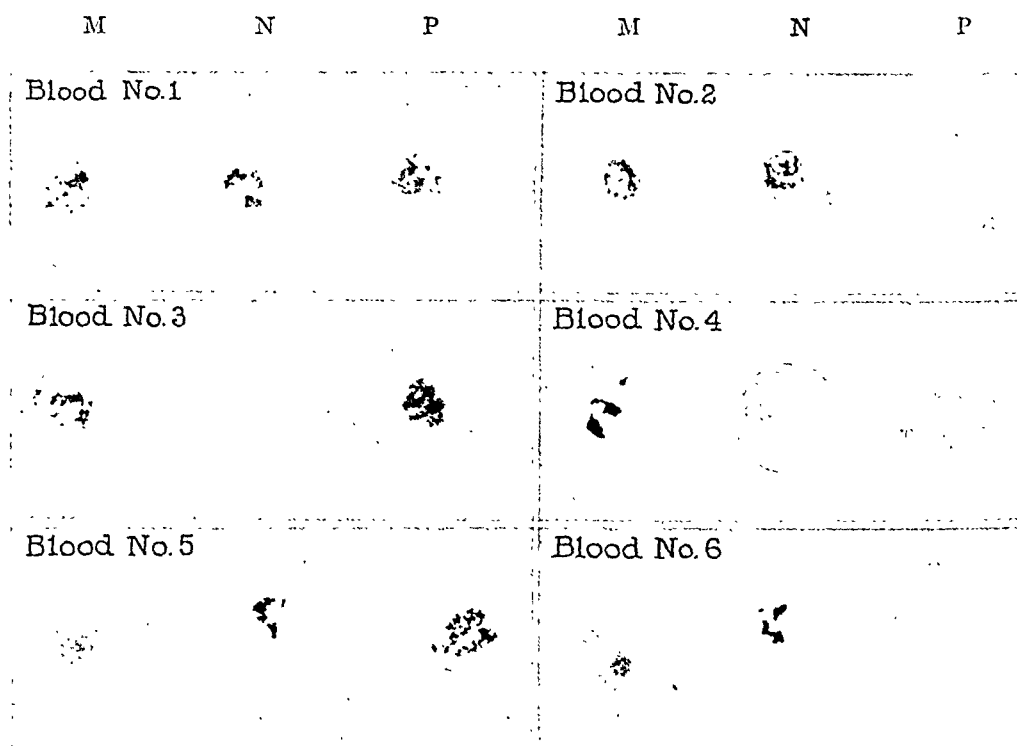


FIG. 1. Six selected bloods of group O tested with agglutinins for M, N, and P; natural size.

The distribution of the property N among the four blood groups is summarized in Table III.

For P our present results do not permit of a similar statistical survey but in general they indicate that there is no characteristic group distribution.

From the presence or absence of three agglutinogens, M, N, or P, there would follow eight possible combinations. Of these six actually

have been found in groups O and A. In groups B and AB some of the rarer types have not yet been found, most likely because of the comparatively smaller number of specimens completely examined.

The six combinations observed are illustrated in the photographs (Figs. 1 and 2). The tests reproduced were made with blood from

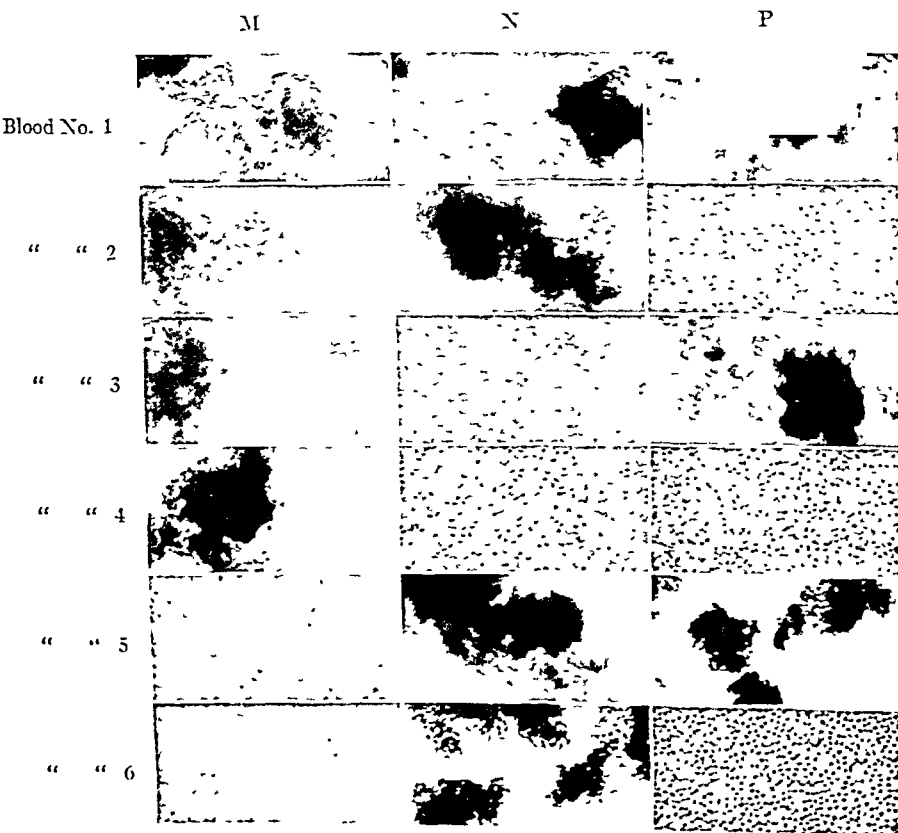


FIG. 2. The same tests as shown in Fig. 1, magnified 1:250.

six selected individuals all belonging to group O. The absorptions and tests for N were carried out at room temperature.

The two combinations not observed are those in which both properties M and N are absent. From the incidence of M- and N- bloods

one would expect an incidence of about 4 per cent bloods lacking both properties. This discrepancy evidences a negative correlation between the two agglutinable properties M and N, a conclusion supported by the fact that invariably M— bloods reacted intensely with the N reagent.

To form a correct judgment of the significance of the phenomena it was of importance to examine in how far they are influenced by variations in the reagents. In the first place a comparison was made of several anti-M immune sera in order to establish whether we were dealing with a definite agglutinable property or whether the results change contingent upon the special serum employed. The following experiments in which five anti-M immune sera were absorbed with the same blood specimen furnish proof for the first alternative (Table IV).

It is evident from the table that the strongly positive reacting bloods are the same regardless of the serum employed. In no case is the reaction of a blood intensely positive with one anti-M serum and negative with another. Identical results have since been obtained with several additional immune sera. Whether there is any disproportion in the strength of the reactions when M+ bloods are tested with a number of immune sera we are unable to decide as yet. With the sera 8 and 20 the reactions are almost uniformly strong or entirely negative. The remaining sera showed slight or weak reactions with some bloods which, doubtless, according to the other tests, lack the property M. These will be discussed presently.

In a second series of tests (Table V) one of the sera, No. 8, was absorbed with five different bloods, one of group O and four of group A, two of which belonged to subgroup AA¹ and two to AA².*

This experiment agrees with the former, for, no matter which blood was used for the preparation of the fluid, the strong reactions occurred with the same blood specimens. Hence it follows that all these reactions involve one sort of agglutinin and its corresponding agglutinin.

The fluid prepared by absorption with blood of group O gives weak or moderate agglutination effects with all the bloods of group A or AB, evidently due to the presence in the immune serum of an agglutinin for A. These reactions are removed by absorption with blood AA¹ while after treatment of the immune serum with blood AA² there is still some agglutination with bloods AA¹ (or AA¹B).

* For the nomenclature of the subgroups see (8, 14).

Similar interfering reactions were encountered not infrequently throughout these studies. In general they were brought about by the presence in the sera of normal or immune antibodies for the group factors A and B, of antibodies acting by preference on bloods of group 0 (Schiff (9)) and by the coexistence in the same immune serum of more than one of the new agglutinins; besides there may be other agglutinogens not yet analyzed. Suitable absorption experiments serve to eliminate such additional antibodies.

TABLE VI, *a*.

Serum 20 diluted 1:15 was absorbed once for 2 hours at room temperature with half its volume of blood lacking the factor M. Some of the fluid obtained was removed and the remainder was divided into two equal parts. One of these two fractions was further absorbed three times at room temperature with half its volume of the M- blood, and the other was simultaneously treated in the same manner but with washed sheep blood; after each absorption a small amount of fluid was withdrawn for the tests. The last absorption was made at 0°C. and the fluid was obtained by centrifuging in a jacket containing ice and water.

The fluids of the first, fourth, and fifth absorptions were titrated in progressively doubled dilutions, using 3 drops of the liquid and 1 drop of 2.5 per cent suspension of M+ and M- blood. Readings were made after the tests were kept 2 hours at room temperature.

Fluid after	Absorbed with blood	Test with blood	Fluid diluted 1:				
			15	30	60	120	240
1st absorption	Human M-	M+	+++	++	+	f. tr.	0
		M-	0	0			
4th "	Human M-	M+	+±	+	f. tr.	0	0
		M-	0	0			
	Sheep	M+	+++±	++	+	0	0
		M-	0	0			
5th "	Human M-	M+	+	f. tr.	0	0	0
		M-	0	0			
	Sheep	M+	+++±	++	+	tr.	0
		M-	0	0			

The question whether the properties M+ and M- are of a qualitative nature was approached by means of repeated absorptions of the immune sera with M- blood. In the experiment recorded in Table VI, *a*, and Table VI, *b*, it was found that the antibody for M could

be gradually absorbed from serum 20, the greatest effect being obtained at low temperature while in serum 72, even after several absorptions, there was only an indifferent diminution of the specific agglutinin, not more marked with human than with sheep blood which was used as a control. Consequently the property M is characterized as a particular agglutininogen according to serological terminology.

Some anti-M sera were found whose specificity could be recognized by different degrees of agglutination when the exhausted sera were tested with the two sorts

TABLE VI, *b*.

An experiment similar to the preceding was made with anti-M immune serum 72. This serum diluted 1:30 required two absorptions with human M- blood for removing the common agglutinins. After withdrawing some of the fluid it was divided in two portions and the experiment was continued as above with human blood and sheep blood. The last absorption was carried out at 0°C. The fluids of the second, fourth, and fifth absorptions were tested as before.

Fluid after	Absorbed with blood	Tested with blood	Fluid diluted 1:				
			30	60	120	240	480
2nd absorption	Human M-	M+	+++	+++	+	+	0
		M-	0	0			
4th "	Human M-	M+	+++	++	+	+	0
		M-	0	0			
	Sheep	M+	+++	++±	±±	+	0
		M-	0	0			
5th "	Human M-	M+	++	±±	±	f. tr.	0
		M-	0	0			
	Sheep	M+	++	±±	±	0	0
		M-	0				

of corpuscles; they were not good for further work because the species agglutinins could not be removed without a simultaneous loss of the specific action.

The action of several (six) anti-N immune sera on a series of bloods and the effect of the exhaustion at room temperature of two immune sera with various bloods were studied in an analogous manner as

described for the property M. Since the strong reactions occurred always with the same bloods the experiments warrant the assumption that here, too, a definite serological factor comes into play. This factor may be subject to some variation as will be discussed presently.

A difficulty was encountered, owing to the fact that on treating anti-N immune sera several times with N-bloods, there was a rather rapid diminution of the agglutinins for N. As a consequence it was not easy to estimate the adequate degree of absorption, although fluids of marked specificity could be prepared repeatedly (see Table I).

TABLE VII.

Each of four anti-N immune sera diluted 1:20 were absorbed three times with one-half volume of pooled blood of four individuals of group A lacking N. One set of the absorptions was performed at room temperature and the other at 37° (water bath). In the latter case the fluids were separated by centrifuging for about 1 minute at high speed in a jacket of warm water (about 50°): At the end of the centrifuging the temperature of the water was 37° or but little below. The fluids were tested with six selected bloods of group A, two of which reacted negatively, two moderately, and two intensely.

Absorptions and tests at room temperature							
Anti-N immune serum	Fluid after	Blood No.					
		806	1010	546	931	851	953
18	1st absorption	tr.	±	++±	++±	+++	+++
	2nd "	0	0	±	tr.	++	++
	3rd "	0	0	0	0	±	±
22	1st "	0	0	++	++±	+++	+++
	2nd "	0	0	±	±	++±	++
	3rd "	0	0	f. tr.	0	+	±
26	1st "	f. tr.	f. tr.	++	++±	+++	+++
	2nd "	0	0	++	+±	++	+++
	3rd "	0	0	+±	+	++	++
61	1st "	tr.	0	++±	+++	+++	+++
	2nd "	0	0	±	+	++±	++±
	3rd "	0	0	tr.	f.tr.	+	+

TABLE VII—*Concluded.*

Absorptions and tests at 37°							
Anti-N immune serum	Fluid after	Blood No.					
		806	1010	546	931	851	953
18	1st absorption	++	+±	++±	+++	++±	++±
	2nd "	f. tr.	0	++±	++±	+++	+++
	3rd "	0	0	++	+±	++±	++±
22	1st "	±	+	++	+++	+++	+++
	2nd "	0	0	++	++	+++	++±
	3rd "	0	0	+±	+	++±	++±
26	1st "	++	+±	++±	++±	+++	++±
	2nd "	+±	+	++±	+++	+++	+++
	3rd "	0	0	++±	++±	+++	++±
61	1st "	++	++	+++	+++	+++	+++
	2nd "	+	±	++±	++±	+++	+++
	3rd "	tr.	f. tr.	+±	++	+++	+++

The technic was improved by carrying out the absorptions and also the tests at 37° or 40° C.* Under these conditions the N antibodies are diminished by repeated absorption with N- bloods at a slow rate (Table VII) and the results are generally satisfactory. On random selection of anti-N sera and absorbing bloods, also weak or moderate reactions are apt to occur with bloods that react negatively when other immune sera or absorbing bloods are chosen. This may be due to quantitative or qualitative variations in the agglutinin N aside from other reactions as discussed above for the property M (p. 766).

The agglutinable property designated as P has not been studied extensively. Doubtless the reactions as presented in Table I are different from those for M and N and are independent of the group agglutinogens A and B; furthermore we found a characteristic distribution of P in white and colored individuals.** But it has not been

* With this method, incidentally, a considerable proportion of stored antihuman blood immune sera were found to contain smaller or larger fractions of N agglutinins.

** See *Proc. Soc. Exp. Biol. and Med.*, 1927, xxiv, 941.

established that parallel results can be obtained with several immune sera which would define a single quality.

A serum of an individual in group B containing an abnormal isoagglutinin was recently described by Ottenberg and Johnson (17). Having had the opportunity to examine this serum, we found in conformity with the authors named, agglutination reactions (of moderate to weak intensity) with numerous bloods of groups O and B. By absorbing this serum with certain bloods A, it was possible to show that the abnormal isoagglutinin acted also on the majority of group A bloods.

These isoagglutination reactions do not coincide with the reactions for M, N, or P.

The observations described open the possibility of making an individual diagnosis of human blood for forensic purposes in cases in which this could not be done hitherto. In preliminary experiments the properties M and N could be demonstrated in blood kept in a dry state (on glass) for several weeks. The method consisted in absorbing specifically reacting fluids with stromata prepared from small amounts (50 mg.) of the dried blood.

Immunization Experiments.—To determine whether the formation of the antibodies described, depends solely on the individuality of the animal used or also upon the antigen injected, the following experiment was carried out: 12 rabbits were injected with blood of the type M+ N— and 6 with blood of the type M— N+, all belonging to group O. Out of the 12 animals after four injections of M+ N— blood there were 1 strong, 1 weak anti-M immune sera, and 5 of rather moderate strength. Two of the latter became strongly active after one or two additional injections. In this series there were 2 or 3 which had a weak action for N. (After a further injection 2 other animals receiving M+ N— blood developed antibodies for N, 1 of moderate and 1 of weak activity.)

Of the 6 animals receiving M— N+ blood all developed immune sera specific for N; 4 reacted strongly and 2 moderately; none of these contained antibodies for M.

The experiment shows that potent antibodies were obtained when the homologous agglutigen was injected but that antibodies of weak activity were also produced by animals which had not received the corresponding antigen. These observations are not exceptional

and similar cases were reported, *e.g.*, by Weil and Felix (18) and Furth (19) with bacilli of the typhoid group (*cf.* Halber and Hirschfeld (20)).

Tests with Blood of Anthropoid Apes.—The presence in the blood of apes of agglutinogens indistinguishable from the human group fac-

TABLE VIII.

*Tests for M in Blood of Primates.**

Anti-M immune serum absorbed with human blood	Chimpanzees										Ourang		Gibbons					Man	
	1	2	3	4	5	6	7	8	9	10	1	2	1	2	3	4	5	M	N
M—	++	++	++	++	++	++	++	++	++	++	++	+	0	0	0	0	0	0	++
M+	0	0	0	0	0	0	0	0	0	0	++	+	0	0	0	0	0	0	0

* The blood of the first 6 chimpanzees, Ourang 1, and Gibbon 1 were examined at the same time.

TABLE IX.

Tests for N in Blood of Primates. Absorptions and Tests Were Made at 37°C. Serum 18 Was Tested after 2 Absorptions (18a) and 3 absorptions (18b).

Absorbed with blood	Immune anti-N serum No.	Chimpanzees				Ourang		Man	
		3	4	5	10			N+	N—
N—	12	tr.	tr.	tr.	tr.	++		+++	0
	18a	+++	++	+++	+++	++		+++	f. tr.
	18b	+	+	++	++	++		+++	0
	22	+	+	0	+	+		+++	0
N+	12	0	0	0	0	++		0	0
	18a	0	±	±	0	++		tr.	0
	18b	0	f. tr.	tr.	0	++		0	0
	22	0	f. tr.	0	0	+		0	0

tors A and B has been shown previously (Landsteiner and Miller (21), *cf.* von Dungern and Hirschfeld (7)). Some experiments were made to establish whether also the new agglutinable properties are to be found in the blood of anthropoids. To account for the existence of agglutinins in the blood of apes that cannot be removed by human

cells,* absorptions had to be made with bloods both lacking and possessing the factor in question; a positive reaction was indicated when agglutination took place in the first but not in the second instance.

The tests for P were negative in the blood of chimpanzees and 1 orang. As to the quality M, it appeared to exist in the erythrocytes of each of 10 chimpanzees, but not in the blood of 5 gibbons (1 *Hylobates lar*, 3 *Hylobates leuciscus*, 1 *Symphalangus syndactylus*) and 2 ourangs (Table VIII). The reactions for N in the blood of chimpanzees were distinctly positive with one of the immune sera, but moderate or faint with two other sera tested (Table IX).

That the properties M and N in the blood of chimpanzees and man are related though not entirely identical is seen from the results with the various anti-N immune sera and from the observation that one anti-M serum very active for human blood acted on chimpanzee blood positively but markedly less so than the other sera.

Of the lower mammals and birds there were examined for the property M: 2 macacus rhesus, 2 vervets, 1 baboon, 1 sapajou, 1 lemur; 1 horse, 4 cattle, 1 sheep, 2 pigs, 1 dog, 1 cat, 25 rabbits, 2 guinea pigs, 2 rats, 1 mouse; 1 duck, 1 chicken, and 1 pigeon. For N only rabbits, 23 in number, were examined (absorptions at room temperature). The tests gave negative results.

DISCUSSION.

In the present studies a method is described which led to the detection of well defined individual differences in human blood in addition to those characterizing the blood groups. On repeated examination of the same individuals the properties were constant. The reactions observed indicate the existence of distinct agglutinable properties. This is substantiated by the fact that for M there are no transitions between positive and negative reactions, since fluids with a titre of 1:64 for M+ blood did not react on M- blood; also blood negative for M has practically no affinity to the antibody of certain anti-M immune sera as shown by absorption experiments at room temperature.

With the two agglutinable properties N and P, an appreciable ab-

* See the tests with orang blood.

sorption effect is brought about at room temperature also by blood negative in the agglutination test. The phenomenon can possibly be explained on the assumption that the antibodies for the particular agglutino-gen and those for human blood in general are not entirely segregated but are partly in some sort of combination. This view is supported by the fact, already mentioned, that certain anti-M sera behave similarly while others stand repeated absorptions. There are other cases which may call for an analogous explanation. It has been shown, i. i., that from some normal human sera of group O, corpuscles A or B absorb not only the homologous but also a part of the heterologous isoagglutinins (8). Similarly agglutinin α^1 of human sera O and B can be removed by bloods of group A which lack A^1 , particularly when the absorptions are made at low temperature.

The ultimate significance of the factors determined by serological reactions is still a matter for discussion and it is not at all certain whether to each factor there corresponds a special compound that might be isolated chemically (14). But there is evidence from a study of families that the agglutinable factors M and N are constitutional properties that are inherited as Mendelian characters.* As to their antigenic nature it is true that the immunization depends largely upon the individual response of the animal but, even so, the experiments indicate that the antibodies can be formed as a result of specific antigenic action.

The division of human blood into only four well defined blood groups was not in harmony with the manifold individual variations that become evident from the experiences on transplantation of normal tissues and tumors. Thus there was some reason to presume that the serological differences of cells and the transplantation specificity are phenomena of a different nature. This gap seems to be bridged by some previous findings (7, 14) and the present studies.

The six types aforementioned, if present, as is likely, in each of the four blood groups and in the subgroups of groups A and AB, differentiate 36 varieties of human blood. This number does not include the variations in the strength of the reactions which may also be determined constitutionally and it is improbable that we succeeded

* See *Proc. Soc. Exp. Biol. and Med.*, 1927, xxiv, 941, and unpublished results.

in detecting all differences which can be demonstrated by means of antibodies derived from rabbits. Possibly other animals when injected with human blood would furnish sera with new specific qualities. As stated already, with a reagent of a different sort, namely an abnormal isoagglutinin in a particular group B serum (Ottensberg and Johnson (17)), reactions were obtained which did not run parallel with those shown by the rabbit immune sera. To be sure this reagent is not available for general use, but still it further doubles the number of human blood varieties that can be differentiated.

Summing up all the known observations on the subject one is led to the opinion that almost every individual human blood may have its characteristic serological features (see Todd and White (5)) as already conjectured by von Dungern and Hirschfeld, although at present there is no actual method which would permit of an individual diagnosis of human blood. Conceivably this end could be achieved by the use of immune isolysins.

The results of studies concerning the heredity of the agglutinable properties and their distribution in populations of different racial composition are reserved for subsequent communications.

The findings dealt with have thus far no direct bearing on the selection of donors in transfusions because of the absence of corresponding agglutinins in normal human sera for the new agglutinogens.

SUMMARY.

A clear-cut differentiation of human blood, aside from the blood groups, could be made by means of special agglutinating immune sera. The observations point to the existence of several agglutinable factors for which no agglutinins are demonstrable in normal human sera. In view of the latter circumstance the results reported do not imply any change in the scheme of the four blood groups.

The body of serological evidence leads to the inference of a high degree of biochemical differentiation among individuals.

Again we are indebted for material used in this study to Dr. C. Floyd Haviland, Superintendent, Drs. I. J. Furman and John R. Knapp, First Assistant Physicians, and Miss Frances W. Witte, Superintendent of Nurses, of the Manhattan State Hospital, New York City.

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THE RELATIVE REACTION WITHIN LIVING MAMMALIAN TISSUES.

X. LITMUS CONSTITUENTS AS VITAL STAINS: THEIR PREPARATION AND RELATIVE USEFULNESS.

BY ROBERT ELMAN, M.D., D. R. DRURY, M.D., AND PHILIP D. McMASTER, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

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The recent use of litmus as a vital stain for mammals (1-3) has emphasized the need for a more precise knowledge of the nature of this dye. We have therefore undertaken the separation and investigation of the coloring matters comprised in commercial litmus with special reference to their use as vital stains.

Rous (1-3) has described in detail the character of the staining obtained in rats and mice after intraperitoneal injections of litmus solutions. The animals tolerate the dye, which colors them markedly, and they may remain stained for many weeks. Some of the tissues are colored diffusely, while the hue of others is traceable to an intracellular segregation of pigment. The observed differences in hue at different situations has great interest since they may be taken to indicate local differences in reaction.

The material used by Rous was prepared from Kahlbaum's cube litmus by extraction with hot water, treatment with acetic acid, and precipitation with alcohol, according to a method given by Sutton (4), similar to one originally described by Mohr (4-6). It contained therefore, according to the statements in the literature, at least three important dyes: erythrolitmin, azolitmin, and erythrolein.

As early as 1840, Kane (7-10) isolated from commercial litmus four constituents: azolitmin, erythrolitmin, erythrolein, and spaniolitmin. He considered the two first mentioned to be the essential coloring matters of the indicator. From time to time other authors have reported the isolation of slightly different pigments, Wartha (11-13) finding what he termed indigotin, and Scheitz (12, 13) a blue coloring matter distinct from azolitmin. On the whole, however,

workers agree that azolitmin and erythrolitmin are the chief sensitive indicator substances present in commercial "litmus," that erythrolein, a relatively insensitive indicator pigment, is always present, while very rarely spaniolitmin is to be found as well, which last can be distinguished from azolitmin only with great difficulty.

Preparation of "Whole Litmus" from Crude Litmus Cubes.

Nearly all of the methods employed for the purification of litmus eliminate from the crude material merely its grosser impurities, notably gypsum. They yield a substance applicable to chemical needs but too complex for physiological experiments. The final product of the method of Mohr (5, 6), or of the procedures given in standard texts on analytical chemistry (4, 14), which we shall term "whole litmus," has been repeatedly analyzed by us and found to contain, as coloring matter, over 90 per cent of erythrolitmin, a little azolitmin, and some erythrolein. There is present in addition a very small amount of gypsum. It was "whole litmus" that Rous used to advantage as a vital indicator.

The "whole litmus" just referred to, while non-toxic when derived from some commercial samples, is highly toxic in other instances. In order to avoid the complication thus introduced, as well as to obtain more precise limiting conditions, we have deemed it best before passing to physiological studies, to isolate and test on animals the individual indicator substances. After trying several methods we have found it best to extract the pigments in the acid state, as follows.

Isolation of the Constituents of "Whole Litmus."

Kahlbaum's litmus cubes were ground to a fine powder, well extracted with hot water, and the insoluble matter removed by centrifugation. The extract was reduced to a convenient bulk by evaporation, and concentrated HCl was added drop by drop to decompose carbonates. When the solution had just turned red, and effervescence had ceased, the volume was measured and 4 cc. of concentrated HCl was added for each 100 cc. of extract. This was then placed on a water bath for 2 or 3 hours, that is to say until no further precipitation occurred. The whole was poured onto a filter paper and the precipitate washed free from acid with hot water and allowed to dry. The dried precipitate was transferred to an extraction thimble of a Soxhlet apparatus and extracted with ether until all soluble material had been removed. The ether was withdrawn

from the Soxhlet, and extraction continued with 95 per cent alcohol until the alcohol came away colorless.

In this way three pigments were isolated. The ethereal solution, when evaporated, yielded an oily red liquid, erythrolein. The alcoholic solution deposited, on evaporation, a bright red powder, erythrolitmin. The brownish red, insoluble residue which remained in the extraction thimble was azolitmin.

The erythrolein needed no further treatment. The filtered alcoholic solution of erythrolitmin was removed to an evaporating dish and carefully dried, washed with ether to remove any remaining erythrolein, and again dried. The azolitmin residue was purified by washing, first with ether, then with alcohol, and dried. Since the pigments in acid state are insoluble in water, and moreover, when powdered and dried do not keep as well as in the alkaline form, which is water soluble, they were converted to the latter. For this purpose thick suspensions were made in water of the dried, powdered pigments in the acid state. Sodium hydroxide in normal solution was added until the indicator became bluish. The material was then evaporated to dryness, and the pigment powdered and kept sealed. For the purposes of vital staining 2 to 5 per cent solutions were made up fresh when desired in 0.9 per cent sodium chloride solution or in water.

"Whole litmus" was prepared, according to the method of Mohr (6, 14), and the product, dissolved in 0.9 per cent sodium chloride solution, was also employed as a vital stain in experiments to be described below.

Characteristics of the Isolated Pigments.

General Characters.—The physical characteristics, solubilities, and colors of the pigment substances generally found in litmus, have been described in detail by previous authors (7, 12, 15). Erythrolitmin and azolitmin are of especial interest to the physiologist for the range of color change of these, which is from red to blue, lies within the presumptive pH range of many living cells. Erythrolein changes hue much less markedly, merely from red, on the alkaline side, to red-orange on the acid, at about pH 6.0 to 5.9. It is therefore only

of interest in that it complicates the use of the whole dye, litmus. In acid solution both erythrolitmin and azolitmin are a clear crimson-red, in alkaline solution blue. In the latter both are dichromatic, being a dark red-violet in very concentrated solutions.

Color Intensity.—The color intensities of erythrolitmin and azolitmin as prepared above are different. A given amount of erythrolitmin will color a solution more deeply than will an equal amount of azolitmin. This has been apparent from comparisons, in a Duboscq colorimeter, of equivalent concentrations of the two dyes, in the alkaline and acid forms, respectively. Both dyes had more than three times the color intensity of "whole litmus."

Colorimeter Readings. Acid Form.—Since the dyes are insoluble in water in the acid form, 0.025 per cent solutions in 10 per cent acid alcohol were compared. All the solutions were reddish orange, of so similar a hue as to afford a good color match.

Five 0.025 per cent solutions of azolitmin were made from specimens of the dye, each prepared from a different batch of litmus cubes. Read in the colorimeter against one another a noticeable variation in color intensity was found, the strongest solution possessing 1.5 times the intensity of the weakest. Similar solutions of erythrolitmin from five specimens of the dye, each from a different source, when compared with one another showed as considerable variations in color strength. A mixture of equal parts of all the azolitmin solutions, compared with a similar mixture of all the erythrolitmin solutions, showed the color strength of the latter to be 1.39 times that of the former.

Alkaline Form.—To study the color intensity of the two dyes in the blue state, comparisons of 0.025 per cent solutions in $N/1$ NaOH were made. The matching of color intensity was easy, despite the fact that the azolitmin was a slaty blue, and the erythrolitmin more azure.

Five alkaline 0.025 per cent solutions of the five specimens of azolitmin were compared in the colorimeter and so too were similar solutions of the erythrolitmin specimens. Of the alkaline azolitmin solutions the strongest possessed 1.6 times the color intensity of the weakest. Among the erythrolitmin solutions a slightly more uni-

form color intensity was found, the strongest being 1.4 times as intense as the weakest. Repeated comparisons of a mixture of equal parts of all the alkaline azolitmin solutions with a similar mixture of all the alkaline erythrolitmin solutions showed the erythrolitmin to be 1.45 times as strong as the azolitmin in color.

The Effect of Differing Concentrations of the Dye upon the Color in Vitro.

Owing to their dichromatism the dyes, erythrolitmin, azolitmin, and "whole litmus," may show colors varying with the concentration, a potential cause of error when they are used as indicators. The magnitude possible to such an error was investigated by comparing the colors of solutions of each of these indicators at differing concentrations. Solutions, varying in strength from 0.005 per cent to 0.05 per cent, were made up in Sørensen's phosphate buffer solutions (16) ($M/15$ primary potassium phosphate KH_2PO_4 and $M/15$ secondary sodium phosphate $Na_2HPO_4 \cdot 2H_2O$), having a pH range from 5.5 to 8.04. In the case of all these indicators the diluted solutions were pure blue at pH 8.0, but with a tenfold increase in concentration appeared red-violet. A shift of the red, toward the alkaline side, had occurred corresponding to a change of 0.2 to 0.3 pH. On the acid side of the range, however, there was no observed difference in the color of the solution with differing concentration.

The degree of color change with alterations in the pH could not be so readily determined in extremely concentrated solutions of the dye as in dilute solutions, but it may, of course, be greater. In the employment of the indicators for tissue study, when they are often found concentrated in intracellular granules, one must take into account possible errors referable to differences in concentration of the dye. For this reason one cannot expect to obtain precise knowledge concerning the pH by the use of the dye. But in connection with this point,—and to anticipate slightly results to be described later,—it may be stated that after injection of the litmus indicators into the mammalian body the differences in color of the intracellular granules are so great that differences in concentration can be but a minor factor in determining them.

Salt and Protein Errors.

The presence of salt or protein in a solution may so affect the color range of indicators that they become of little value for determining its hydrogen ion concentration. The salt and protein errors of litmus and azolitmin have been studied in the past and found to be great (17); but the possibility presents itself that these errors may not be shared by erythrolitmin, the constituent of litmus employed for purposes of vital staining in the studies to be reported in a companion paper. We therefore determined the magnitude of the errors of this dye. These errors were found to be large. A specimen protocol follows which illustrates the magnitude of the salt error in solutions containing differing concentrations of sodium chloride.

TABLE I.

Concentration of NaCl	Apparent pH color	Actual pH (electrometric)	Difference (salt error)
<i>per cent</i>			
.2	7.21	6.99	.22
.4	7.21	6.93	.28
.8	7.21	6.85	.36
1.6	7.16	6.70	.46
3.2	7.12	6.58	.59
4.8	7.08	6.41	.65

Salt Error of Erythrolitmin.—Sørensen's $M/100$ phosphate buffer solutions were prepared, having a pH range from 5.5 to 8.34. To 10 cc. of each in a series of tubes of equal bore 0.3 cc. of a 0.1 per cent solution of erythrolitmin was added. In a companion series of tubes 5 cc. of $M/50$ phosphate buffer of pH 7.0 and 0.3 cc. of 0.1 per cent erythrolitmin solution were placed. To each of this latter series of tubes sufficient quantities of sodium chloride in 5 cc. of water were added to yield 0.2 per cent, 0.4 per cent, 0.8 per cent, 1.6 per cent, 3.2 per cent, and 4.8 per cent sodium chloride solutions in the total volume of 10.3 cc. The concentration of buffer in these tubes became therefore like that of the control series, $M/100$, and the amount of erythrolitmin present in all was similar. The addition of sodium chloride brought about a pronounced change of color, the solutions appearing more blue with each increase in the salt content, as compared with the controls. Electrometric determinations of the hydrogen ion concentration of all the solutions were then made. As the table given below shows, the solutions containing salt appeared bluer, that is to say, more alkaline than their true pH would warrant.

From this experiment it is obvious that the "salt error" of erythrolitmin is large. It is of importance to note that the error is toward the alkaline side, that is to say, in the opposite direction from the error of the phthalein indicators.

Protein Error of Erythrolitmin.—Erythrolitmin shares also the disadvantage of litmus and of azolitmin in that its protein error is large. This, like the salt error, causes the indicator to appear more blue in a solution containing protein than it should from the actual hydrogen ion concentration. For the experiments we employed blood plasma and egg albumin solutions. A specimen protocol will be detailed.

Sufficient $M/15$ phosphate buffer, pH 6.47, was added to clear rabbit plasma to give the mixture $M/50$ concentration of this buffer. Air was bubbled through for 30 minutes to remove CO_2 and then for another half hour a mixture of equal parts of air and hydrogen. The addition of a small amount of this plasma, 1 to 9 cc. of $M/15$ buffer solution, pH 6.9, containing erythrolitmin in the red-violet stage, caused a change in the color to a pure blue.

To determine the protein error of erythrolitmin it was deemed best to make up plasma-indicator mixtures in such a way that their final color would be neither blue nor red but violet, as is erythrolitmin alone in buffer solutions of hydrogen ion concentration between pH 6.9 and 7.4. Within this narrow range a change in color can be noted with each difference of 0.2 pH. Test solutions were therefore made up of a total bulk of 10.3 cc., in each of which was 0.3 cc. of 0.1 per cent erythrolitmin solution and varying amounts of $M/100$ phosphate buffer, pH 5.59, and of a mixture in equal parts of buffered plasma, $M/50$, and of distilled water free from CO_2 (see Table II). These mixtures contained, respectively, 10 per cent, 20 per cent, 40 per cent, 50 per cent, and 60 per cent of plasma and the concentration of buffer in all was the same, a shade less than $M/100$.

For color comparison a series of $M/100$ phosphate buffer solutions, having a pH range from 5.04 to 8.34, was used and to 10 cc. of each, 0.3 cc. of the 0.1 per cent erythrolitmin solution was added. Upon adding erythrolitmin to plasma one encounters in addition to the protein error, another error due to the salt present with the protein. To determine the share of this additional source of error, "control solutions" were made up, each containing in the same concentration of phosphate buffer the amount of salt presumably added to the "test solutions" with the plasma. This was done by employing Ringer's solution minus its sodium carbonate content, together with the necessary amount of $M/15$ phosphate buffer solution to bring the buffer concentration of the total mixtures to $M/100$. In this way "control solutions" were obtained which were nearly like the "test solutions" except in the respect that the latter contained protein. In order to find "control solutions" which matched the violet "test solutions," it was necessary to make up several series. For as the amount of plasma in the

TABLE II.
Erythrolitmin Color as Influenced by Blood Plasma Constituents.

1	2	3	4	5	6
"Test solution"	"Control solution" containing the plasma equivalent in salts	Color comparison with erythrolitmin in phosphate buffer	Error due to plasma (salt and protein error). pH in Column 3 minus pH in Column 1	Error due to the salt equivalent of the plasma. pH in Column 3 minus pH in Column 2	Error due to protein alone. pH in Column 4 minus pH in Column 5
1 0 cc. plasma, M/50 phosphate buffer content	1.0 cc. Ringer's solution				
1.0 cc. water	7.5 cc. water				
8.0 cc. M/100 phosphate buffer, pH 5.59	1.5 cc. M/15 phosphate buffer, pH 6.9				
0.3 cc. 0.1 per cent erythrolitmin solution	0.3 cc. 0.1 per cent erythrolitmin solution	Color of both solutions matches that of erythrolitmin in M/100 phosphate buffer, pH 7.1	pH 0.76	pH 0.23	pH 0.53
10.3 cc. buffer concentration M/100	10.3 cc. buffer concentration M/100				
pH electrometrically 6.42	pH electrometrically 6.95	pH electrometrically 7.18			
2.0 cc. plasma, M/50 phosphate buffer content	2.0 cc. Ringer's solution				
2.0 cc. water	6.5 cc. water				
6.0 cc. M/100 phosphate buffer, pH 5.59	1.5 cc. M/15 phosphate buffer, pH 7.4				
0.3 cc. 0.1 per cent erythrolitmin solution	0.3 cc. 0.1 per cent erythrolitmin solution	Color of both solutions matches that of erythrolitmin in M/100 phosphate buffer, pH 7.7	pH 0.92	pH 0.23	pH 0.69
10.3 cc. buffer concentration M/100	10.3 cc. buffer concentration M/100				
pH electrometrically 6.67	pH electrometrically 7.36	pH electrometrically 7.59			

3.0 cc. plasma, 1/50 phosphate buffer content	3.0 cc. Ringer's solution	Color of both solutions matches that of erythrolitmin in 1/100 phosphate buffer, pH 8.04	pH 1.16	pH 0.33	pH 0.83
3.0 cc. water	5.5 cc. water				
4.0 cc. 1/100 phosphate buffer, pH 5.59	1.5 cc. 1/15 phosphate buffer, pH 7.8				
0.3 cc. 0.1 per cent erythrolitmin solution	0.3 cc. 0.1 per cent erythrolitmin solution				
10.3 cc. buffer concentration 1/100	10.3 cc. buffer concentration 1/100	pH electrometrically 8.01			
pH electrometrically 6.85	pH electrometrically 7.63				

"test solutions" was increased a greater and greater color shift toward the blue side occurred (see Table II).

The actual hydrogen ion concentration of all the solutions was determined electrometrically.

It was evident from such findings as are given in the table that the protein error of erythrolitmin is very large. When mixtures of the indicator are made with plasma or egg albumin, containing salt as well, the apparent error is still greater, for both salt and protein cause a change in the color of the indicator in the same direction, that is to say toward the alkaline side. As a result of this combined error a protein solution which is at about pH 7.0, that is to say, neutral, may appear frankly alkaline upon the addition of erythrolitmin, that is to say, blue.

When to 10 cc. of rabbit's plasma rendered CO_2 free and buffered as described above with sufficient M/15 phosphate buffer to give the mixture M/50 buffer concentration, 0.3 cc. of 0.1 per cent erythrolitmin solution was added, the color of the mixture matched that of erythrolitmin alone in phosphate buffer M/100 of pH 8.34. The actual pH of the protein mixture determined electrometrically was pH 7.20 and that of the buffer solution 8.19. The colorimetric reading was therefore in error by pH 1.0. In M/100 buffer solutions the color of erythrolitmin at pH 7.2 is red-violet, at 8.34 pure blue.

It has been noted elsewhere (1, 2, 3) that litmus segregated by living cells out of body fluids dyed blue with it is held in the red form. This is scarcely what one would expect were the intracellular reaction neutral or slightly alkaline for the influence both of protein and of salt would tend to render the litmus blue under such conditions. It is a matter of interest therefore to know the actual hydrogen ion concentration of protein mixtures rendered just sufficiently acid to bring the indicator to the red color it assumes in intracellular granules.

The protocol of one experiment on the point out of a number will be given.

To 3 cc. of buffered plasma, 1 cc. M/3 KH_2PO_4 solution (pH about 4.5) was added and 0.3 cc. of 0.1 per cent erythrolitmin solution. The resulting color was violet, not red. To the mixture phosphoric acid M/15 was added in just sufficient quantity (0.6 cc.) to bring about a brick red color like that of the erythrolitmin-stained granules frequently seen within tissue cells. The color of this

mixture matched that of erythrolitmin in $N/15$ phosphate buffer of pH 5.91. Electrometrically the pH of the plasma solution was found to be 4.81.

In connection with such findings it is to be recalled that the concentration error of erythrolitmin is in the opposite direction to that of the salt and protein errors, that is to say, the more concentrated the dye the more does it tend to appear red. It follows that an intracellular granule containing the dye in concentrated form need not have as great an acidity as that prevailing in the experiment above in order that it shall appear red.

From the foregoing results taken together it is obvious that in vital staining with erythrolitmin, one must content oneself ordinarily with the determination of relative differences in pH.

The Donnan Equilibrium Has No Effect upon the Distribution of the Litmus Derivatives.

A dye taken up by tissue cells becomes subject to the forces incident to the presence of protein substances. Our finding, that some changes in the color of the dyes result from changes in their concentration, made it important to study the influence of hydrogen ion concentration on the distribution of dye between fluid and protein, in mixtures of the two. A series of solutions were prepared containing powdered isoelectric gelatin and varying amounts of acid and alkali. "Whole litmus" or erythrolitmin was added to each. 20 hours later the fluid was separated by filtration and the amount of dye in it measured colorimetrically. All the solutions were found to contain the same proportions of the indicator. The conditions set up by the Donnan equilibrium did not change the relative distribution of the dye at the various hydrogen ion concentrations. From this finding it would seem that the probability of an error, caused by concentration changes due to the presence of protein, is slight.

A series of acid and alkaline mixtures were made up in 0.9 per cent NaCl solution, using $N/1$ HCl and $N/1$ NaOH in proper quantities to give $N/5$, $N/20$, $N/40$, $N/80$, $N/160$ to $N/1280$ acid in saline, and $N/80$, $N/160$, $N/320$, $N/640$, and $N/1280$ alkaline in saline. To 50 cc. of each of these 2 gm. of powdered isoelectric gelatin was added and 1 cc. of a 1 per cent solution of "whole litmus," just sufficiently alkaline to be in the blue state. The mixtures were each shaken 1 minute, packed in ice, and allowed to stand with the gelatin completely submerged in the fluid

for 20 hours. The litmus solution gave a red color to the gelatin and a blue to the supernatant liquid of the N/640 and N/1280 acid solutions immediately upon its addition to them; but after standing the color of the gelatin and supernatant liquid was uniform. The N/160 and N/320 NaOH solutions seemed to be close to the neutral point for the indicator, whereas the N/80 NaOH solution was alkaline to it, as shown by the distinctly blue color, and the N/640 NaOH solution acid, appearing red.

The supernatant liquids were separated from the gelatin by rapid suction filtration and the amount of dye present in each was estimated by adding to each 0.6 cc. of 40 per cent NaOH to convert the color to a strong blue to be read in a Duboscq colorimeter against a standard. For the latter purpose there was employed a solution containing 1 cc. of 1 per cent "whole litmus" to 50 cc. of saline with 0.6 cc. of 40 per cent NaOH. It will be seen that the amount of indicator equalled the total added to each of the gelatin mixtures.

The distribution of litmus was as follows:

Acid.

	N/80	N/160	N/320	N/640	N/1280
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
In supernatant liquid	54	53	52	47	49

Alkaline.

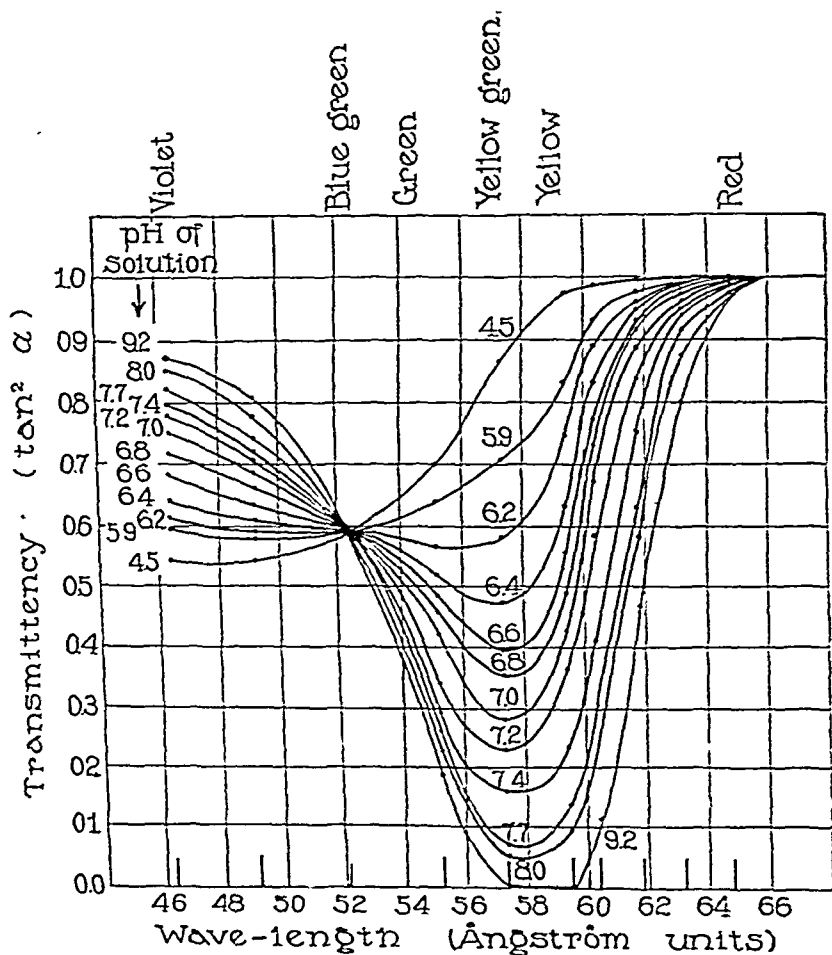
	N/1280	N/640	N/320	N/160	N/80
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
In supernatant liquid	49	56	52	61	58

The concentration of chloride ion in the supernatant liquids was found to be about the same in all. There was proportionately a little more in the gelatin. The determinations were made by adding an excess of silver nitrate to the solutions and titrating the amount of the excess with potassium thiocyanate, with iron alum as the indicator.

The behavior of erythrolitmin, studied in the same way, was found to be completely analogous to that of "whole litmus."

Indicator Properties of Erythrolitmin as Shown by the Spectrophotometer.

The property of certain dyes which enables them to serve as indicators is their ability to absorb different proportions of light in a given part of the spectrum at different hydrogen ion concentrations. The



TEXT-FIG. 1. The absorption of light by 0.01 per cent solutions of erythrolitmin, in buffers, at various hydrogen ion concentrations. The amount of light transmitted by the solutions is plotted along the ordinate, and along the abscissa, in Ångström units, light wave-lengths. Each curve records the variations in light absorption, in various regions of the spectrum, by a single solution of erythrolitmin at a definite pH. The regions of the spectrum at which readings were made are designated on the chart. Within the blue region of the spectrum, the more alkaline the solution, the more blue is transmitted. On the red side of the spectrum, the more acid the solution, the more red comes through.

phenomenon can be readily measured with the spectrophotometer when the instrument has been set to permit the passage of light of known wave-lengths, while allowing light of but one wave-length to pass at any given time. Studies of various dyes have been made in this way, and Brode (18) has recently applied the method to synthetic indicator substances, phenol red, brom cresol blue, and others.

A study of erythrolitmin and azolitmin with the spectrophotometer is of value in that it shows graphically the manner in which the substances act as indicators. It has proved furthermore that they are pure indicators, on a par in this respect with the synthetic dyes examined by Brode.

Four series of Sørensen's phosphate buffer solutions (16) were prepared, each containing 0.02 cc. of 5 per cent azolitmin or erythrolitmin to every 10 cc. As a control solution uncolored buffer was employed. Both were placed in glass cells 10 cm. long. The amount of light absorbed at certain arbitrary points on the spectrum was measured for each solution and a series of curves prepared. Those for erythrolitmin are shown in Text-fig. 1.

The instrument used was of the type first designated by König (19), modified by Martens (20), and made by Franz Schmidt and Haensch (Berlin). The apparatus is briefly described by Sheppard (21). A more detailed description can be found in the paper by Martens and Grünbaum (22), and by Grünbaum (23).

Briefly, the instrument provides for the passage of 2 equal beams of light from a single source, which pass through the solutions to be studied, then through 2 adjustable slits and a collimating lens and are finally refracted by a single flint prism. The emerging beams pass into the movable arm of the instrument which, by a graduated screw, can be placed at any angle thus admitting the light from any desired portion of the spectrum. Here the 2 refracted beams are polarized by a Wollaston calcite prism, passed through a biprism and brought to a focus by a telescope lens.

At the eyepiece 2 images, 1 from each slit, are seen polarized at right angles to each other. By rotating the crossed Nicol prism placed in the eyepiece the intensity of the light in each half of the field is reciprocally altered. Before making any observations the instrument is so adjusted that a movement of the Nicol prism to 45° from the zero point illuminates the 2 fields equally. This was also found to be the case, as it should be, at a rotation of 135°, 225°, and 315°.

$$\text{Calculation: } \frac{S}{C} = \frac{\sin^2 a}{\cos^2 a} \quad \text{Where } S = \text{light transmitted through solution containing the dye.}$$

$$\frac{S}{C} = \tan^2 a \quad C = \text{light through the control solution.}$$

$$a = \text{angle of deviation (from } 45^\circ\text{).}$$

The amount of light transmitted through the colored solution will vary from 0, when all light is absorbed, to 1, when none is absorbed, that is to say, when the same amount of light comes through on the 2 sides. The light used was a Nernst filament set in series with a resistance coil. The readings of angular deviation were converted to Ångström units, by calibrating the instrument with known spectral lines and interpolating between.

The accompanying figure represents the findings for erythrolitmin. Azolitmin gave practically the same curves. The amount of light transmitted by the solutions is plotted along the ordinate and is simply the reading of $\tan^2 a$, subtracted from 1, since $\tan^2 a$ represents percentage of absorption. Along the abscissa are plotted the wave-lengths which correspond to the different parts of the spectrum in which the readings are made. Each curve records the variations in absorption of a solution of given pH and it is seen that they fall into a regular order with relation to each other.

It is of great significance that the curves of light absorption in solutions of different pH all cross at a single point, for this is characteristic of pure indicator dyes (18). In this case the point lies at a wave-length of 5250 Ångström units, which corresponds to a blue-green. Regardless of pH the proportion of light transmitted at this point is the same, 58 per cent. On the blue side of the spectrum, the more alkaline the solution, the more blue comes through. On the red side of the spectrum, the more acid the solution, the more red comes through.

The Relative Worth of the Litmus Dyes as Vital Stains.

Rous has recently used litmus as a vital stain in rats and mice (1, 2, 3). For weeks after intraperitoneal injection the tissues of these animals are still colored with it and it has retained its indicator properties. There are numerous differences to be seen which are presumably indicative of local differences in the reaction. We have made an effort in the course of the present work to throw more light on some of the phenomena observed after injection of litmus by comparing with them the effects of injection of the constituent coloring matters.

The litmus used by Rous (1) contained at least the three coloring matters, erythrolitmin, azolitmin, and erythrolein. *A priori* it would seem that the

heterogeneity of litmus might be disregarded when it is employed as a vital stain because of the circumstance recognized by others before us (15) that one of the constituents, erythrolitmin, makes up 90 per cent or more of the whole dye. But many authors have considered azolitmin the active principle of litmus, and conceivably this may have been true of the preparations with which they worked. Our material (Kahlbaum's litmus) which was the same that Rous employed yielded over 90 per cent of erythrolitmin, as already stated. Both azolitmin and erythrolitmin are indicators *in vitro* with practically the same virage and absorption bands, as we have pointed out, but the latter is a more intense dye and, as will be shown below, is more suitable for experiments *in vivo*. The third dye constituent of litmus, erythrolein, is a poor indicator for tissue study since the color change is merely from red to red-orange.

Procedure.

"Whole litmus," erythrolitmin, azolitmin, and erythrolein were separated out of litmus purified as already described, and injected into the peritoneal cavity of rats and mice. The individual dyes like the "whole litmus" were made up in 2 and 5 per cent solutions in 0.9 per cent sodium chloride solution. Sterilization was effected by heating test-tubes partly filled with the solutions in boiling water for 30 to 60 minutes.

The dyes were given with aseptic precautions. Graded doses were administered to many series of animals and special note was made of the rapidity and depth of the tissue staining which ensued. Special cages were employed for the collection of total urine. At various intervals, from half an hour to 10 days after the injections, the animals were put lightly under ether and guillotined. Autopsy was done at once, and the tissues examined. Fresh sections of the kidney, and frequently of other organs, made with the Valentine knife, were studied, and frozen sections were examined later. The kidney findings will form the subject of a succeeding communication. Staining of the tissues was best accomplished by injecting the dyes twice at an interval of 48 hours, killing the animal 2 days after the last injection.

Experimental Findings.

Erythrolein.—No generalized staining followed the administration of this dye. Nearly all of it was soon secreted into the urine which assumed in consequence an intense red color that failed to change notably on the addition of alkali. Moreover, erythrolein proved strongly toxic, causing death in doses of $\frac{1}{2}$ mg. per gm. of body weight.

Azolitmin.—The 5 per cent solution of azolitmin in water or physiological saline proved to be thick and viscid. The indicator was absorbed slowly from the abdominal cavity and much of it could be

found there after 24 hours or more. As a consequence animals were not well colored after the injection of $\frac{1}{4}$ mg. of the dye per gm. of body weight. To obtain satisfactory staining as much as $\frac{1}{2}$ mg. per gm. of animal was necessary, an amount which proved toxic and usually lethal.

Even the largest non-toxic dose stained the kidney but poorly. On the day following a single injection the glomeruli appeared intensely blue from the presence of the dye in the capillary tufts, and blue streaks in the kidney cortex represented the blood vessels in the midst of the pale, unstained parenchyma. The general staining of other organs was slight, but became definite after a second administration of the dye. When examination was made 2 days thereafter, the kidney contained much of the indicator as compared with the amount elsewhere. It was present in the tubule cells for the most part as large red granules though some cells contained blue ones.

Erythrolitmin.—Erythrolitmin has proved far superior to azolitmin or "whole litmus" as a vital stain. *In vitro* it exhibits a greater color intensity than azolitmin, as already demonstrated, and when injected into animals it is much less toxic, doses of $\frac{1}{2}$ mg. per gm. of body weight being well borne. A generalized blue staining with the dissolved dye is caused by but $\frac{1}{4}$ mg. of erythrolitmin per gm. of animal. The injection of this amount causes a pronounced transient bluing of the body. The dye is employed to advantage in 2 per cent solution.

Within an hour after the injection of erythrolitmin into rats and mice blue areas appeared on the pads of the feet and in the ears of the injected animals, and within 2 to 3 hours the entire body was colored blue. After 24 hours little of the indicator could be recovered from the peritoneal cavity, practically all having been absorbed. At this time the animals were diffusely colored and the dye could be found in almost every tissue. Later much of it became segregated in granular form within cells. It is unnecessary to go into the findings in detail since they are identical with those recorded by Rous (1, 2, 3) for rats and mice injected with "whole litmus." After repeated doses the staining became intense and at autopsy most of the tissues appeared highly colored, the kidneys in particular containing large amounts of the dye. Within the tubule cells in

certain regions were granules stained a brilliant red, while in others they were blue, as observed by Rous (1).

The urine voided shortly after the injection was usually red, but, unlike the red urine found after injections with erythrolein, turned blue upon the addition of alkali. But soon excretion of the dye ceased, the urine becoming colorless and remaining so.

"*Whole Litmus.*"—In the course of experiments, to be described in a later paper, numerous rats and mice were injected with "whole litmus" and examined after various intervals of time. The material proved toxic as compared with erythrolitmin and the staining of the tissues effected by it was less intense. The toxic effects would seem from our findings to have been due in considerable part to substances other than the indicator dyes. The various batches of "whole litmus," prepared by the same method, differed greatly in toxicity. Doses of $\frac{1}{2}$ mg. per gm. of body weight of animal were often tolerated well, but at other times proved fatal. The specimens varied much in color intensity as well. Some contained much erythrolein, others little, and differences in the content of azolitmin and erythrolitmin were also found. The first urine collected after injection with "whole litmus" was red, often intensely so, but the addition of alkali caused no change to blue. From the characters of erythrolein, above described, one may conclude that the red color of the urine was due to the excretion of the erythrolein portion of the "whole litmus." Some part of the relative toxicity of the latter may also be ascribed to its content of this pigment.

DISCUSSION.

Caution must be exercised in the interpretation of findings with erythrolitmin and azolitmin when used as vital stains, despite the fact that the light absorption phenomena of these dyes, when prepared as described above, place them on a par as concerns purity with synthetic indicators such as phenol red (18). Both of the dyes are colloidal and they tend to become segregated within the cells in granules. They are dichromatic as well, and because of this may give erroneous information when variously concentrated within cells. The total error from this source and from the presence of salt and protein is smaller, however, than one might expect. And our experi-

ments on the distribution of erythrolitmin and "whole litmus" between a protein and the fluid bathing it, as exemplified by gelatin and water, fail to demonstrate any important influence of the Donnan equilibrium.

While no accurate inferences concerning the absolute pH within living tissues can be drawn through the use of these dyes, changes in the relative reaction can be followed with ease. It is with this in mind that we have studied the indicators.

It is evident from our experiments that one may ascribe the tissue staining described by Rous (1, 2) almost wholly to the erythrolitmin content of the "whole litmus" that he employed.

SUMMARY.

We have devised methods for the separation and isolation of the important indicator constituents of litmus, azolitmin, and erythrolitmin, with a view to employing them as vital stains. Analysis of the color intensities of these dyes shows slight differences in them, azolitmin being the weaker pigment, weight for weight. Study of a third coloring matter, erythrolein, which exists in litmus has shown it to be an unsatisfactory indicator, and toxic for animals.

Analyses with the spectrophotometer of the absorption of light by erythrolitmin and azolitmin, prepared by our methods, and tested over a wide acid-alkali range, show them to be pure substances, comparable in this respect with synthetic indicators.

The errors in the interpretation of the indicator phenomena on vital staining, which are incident to changes in the concentration of the dyes, are so slight as to be negligible. The salt and protein errors on the other hand are large. The factors responsible for the Donnan equilibrium fail to influence the distribution of the indicators between fluid and gelatin.

Erythrolein was found useless when employed for vital staining, and azolitmin proved unsatisfactory since it colors poorly and is toxic. But erythrolitmin can be used to great advantage. It is readily absorbed, and in non-toxic doses stains intensely. The range of pH at which it changes from red to blue fits it for the demonstration of changes in the reaction of living tissues. By reason, however, of the

salt and protein errors to which it is liable, the pH cannot be accurately ascertained.

Intravital staining with erythrolitmin yields results similar to those following injection of purified "whole litmus."

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THE RELATIVE REACTION WITHIN LIVING MAMMALIAN TISSUES.

XI. THE INTRACELLULAR REACTION OF THE KIDNEY EPITHELIUM AND ITS RELATION TO THE REACTION OF THE URINE.

By PHILIP D. McMASTER, M.D., AND ROBERT ELMAN, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

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In the past the injection of dye substances into the circulation of living animals has yielded much information about the functions of certain regions of the kidney (1-7). Recently Rous (8-11) has reported studies of the relative reaction of living cells carried out by the use of various indicator dyes. Some of his methods are well adapted to a study of the reaction within kidney cells and he has observed that litmus when segregated within the renal epithelium is in some places red, in others blue, a fact suggestive of marked functional differences. In the work here to be reported this observation has been followed up and we shall record certain changes in the reaction within cells lining the renal tubules of mammals, which changes accompany and are largely, if not entirely conditioned by alterations in the functional activities of these cells.

Previous Literature.

The older literature on vital staining of the kidney has been reviewed by Cushny (12) and recently by Marshall and Crane (13). The elimination of dye substances by the glomeruli has been demonstrated by Wearn and Richards (14-16) and by Bieter and Hirschfelder (17). Workers are at one concerning it, but there has been much difference of opinion as to the significance of the presence of dye substances within the tubule cells. Heidenhain (1) took their presence to indicate a removal of the dye by these cells out of the blood and into the urine, a secretory activity in other words; but many more recent workers have looked upon it (6, 12) as a result of absorption from the tubule lumen. Recently Marshall and Vickers (18) and Marshall and Crane (13) have reported studies of the excretion of phenol red which they take to indicate a direct secretion of the dye by the tubular epithelium.

Indicator dyes have been much used for the study of kidney function in general but very little for that of the reaction prevailing within renal cells. Long ago Dreser (19, 20) found acid fuchsin in the acid state in the cells of the convoluted and straight tubules of the frog following repeated injections of the dye, and Grützner (21) reported that the glomeruli were stained with indigosulfonate in the acid form under conditions of asphyxia. Rous (22) noted that the color of the renal cortex of rats and mice injected with various of the phthalein indicators was often such as would indicate a pronounced degree of acidity; and more recently Edwards and Marshall (23) have noted that phenolsulfonephthalein when present in the convoluted tubule cells of the dog kidney has the yellow color of acidity. Stieglitz (7) had previously employed azolitmin, neutral red, and sodium alizarinate in a study of changes in the reaction of the renal tissue. He injected the dyes directly into the blood stream of rabbits and dogs and killed the animals within a few minutes. He took the coloration to indicate that the reaction prevailing within the kidney cortex is alkaline when an acid urine is being secreted and *vice versa*. No histological evidence is given in his paper.

In the experiments here to be described we have studied the reaction within kidney cells by means of one of the pigmented ingredients of litmus, erythrolitmin (24-27), which is taken up and held by these cells for considerable periods. Rous, in his papers on the relative reaction of mammalian tissues (8-11, 22) noted that the tubule cells of the rat kidney, after an intraperitoneal injection of litmus, contained abundant granular deposits of the dye, which persisted for weeks and even months after the injection. Since the intracellular material was found on test to have retained its indicator properties it was evident that a study of the reaction within the cells could be made under varying physiological circumstances.

The Suitability of Erythrolitmin for Kidney Cell Studies.

More than 90 per cent of the purified "whole litmus" employed by Rous consisted of erythrolitmin, and it is to this ingredient that the staining he obtained is to be attributed. Erythrolitmin is far superior to azolitmin or to "whole litmus" for purposes of vital staining, being practically non-toxic, and its change from red to blue occurs within the range of hydrogen ion concentration encountered in acid and alkaline urines (12, 24). These facts, coupled with the finding that intraperitoneal injections of the substance did not cause nephritis, as evidenced histologically by sections of the kidney and by the absence of albumin in the urines of the injected animals, led us to employ

it for the present work according to a method described below. Only pure erythrolitmin was used, save where mention to the contrary is made. After intraperitoneal injections the dye is segregated within the tubular epithelial cells everywhere from the glomerulus to the collecting tubule, and shows striking regional differences in color under differing conditions of functional activity. A diffuse staining of the intracellular granules takes place, not a mere surface accumulation of dye such as some authors believe occurs (11) who have dealt with renal epithelium stained in other ways.

Character of the Staining.

The exact situation of dye substances segregated within cells has been the subject of great dispute. The indicator material retained within cells after injections of "whole litmus" is sometimes concentrated upon the surfaces of cell granules and in other cases is diffusely distributed within the latter (8, 9). The colored granules which can be squeezed from renal epithelial cells, after an intraperitoneal injection of erythrolitmin, show in our experience a diffuse distribution of the stain now in dilute form and again greatly concentrated. Many granules spread like masses of a soft gel when pressure is exerted upon the cover slip, but others resist such pressure as can readily be brought to bear and retain their form. There are never any appearances that would suggest a precipitation of the dye. Always it is evenly distributed and the granules are translucent.

Procedure.

Rats of 80 to 200 gm. weight were given intraperitoneal injections of a 2 per cent solution of erythrolitmin in 0.9 per cent sodium chloride solution. The material had been prepared according to a method described in the foregoing paper (24). For every gm. of body weight $\frac{1}{2}$ to $\frac{1}{4}$ mg. of the dye was given and the injection was repeated once or twice at 48 hour intervals. The result was an excellent staining of the kidney. As a rule the organ was examined 48 hours after the last injection, but the findings were much the same when the interval was several hours shorter. At the time for examination of the kidneys the animals were given a whiff of ether, guillotined, stretched out upon a paraffin slab, and submerged in paraffin oil. The kidneys were removed with all possible speed, decapsulated, and sectioned with a Valentine knife while still under the oil. The whole process required often no more than 45 seconds to 1 minute. The sections, protected by oil, were placed on mica slides, covered with thin slips of the same material, and

ringed with paraffin. As these sections were often about 100μ thick, and on the average about 150 to 175μ , observations on the color of the dye granules within the kidney tubule cells could readily be made. Frozen sections also were made and studied to determine the distribution of the dye irrespective of its color. On freezing the tissues, the granules previously diversely colored, red, blue, or violet, became all of one hue, violet, as result of the process of freezing and immersion of the sections in water before mounting. The granules within the cells of fresh sections made with the Valentine knife, on the other hand, did not change color during the first few minutes of observation, often maintaining their original hue for 20 minutes or more, a fact which may be taken to indicate that the coloration was identical with that existing during life.

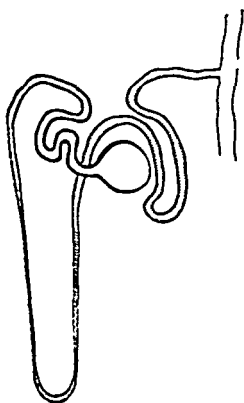
Findings in the Normal Rat Kidney.

In the gross the stained kidneys appeared a dark purple-red. Under the low power of the microscope the outer zone of the cortex appeared blue at first sight, the inner zone red, and the medulla unstained. Closer inspection showed, however, that there were marked local differences underlying the prevailing hues. Much of the renal cortex was unstained. The glomerular tufts showed no litmus but were well filled with blood cells of the natural hue. The endothelial lining cells of the glomerular capsule and particularly those near the entrance of the uriniferous tubule always appeared a deep brilliant blue, containing the dye either in very fine granules, or at times in what seemed to be the diffuse form. Wherever the proximal convoluted tubule could be seen leading off, the cells lining it contained the dye in bright blue granules, which were so abundant as to lend a blue hue to the cells. In most sections this picture was readily to be observed. The glomerular tufts appeared as though in the cup of a blue crescent with a blue stem.

Wherever the eye could follow a single tubule far toward the renal pelvis, the color of the granules contained in the epithelial cells was observed to vary with the region. Near the glomerulus the color was blue, and further down violet, while it was red a very little further on. In that short segment where the tubule cells in general appeared violet, owing to their content of granules having this color, individual epithelial cells were to be found containing granules some of which were brilliant red and others bright blue. In every section there were portions of tubules occupying the zone intermediate between the outer and inner cortical areas of the kidney, that could not be identified definitely. These usually contained violet granules.

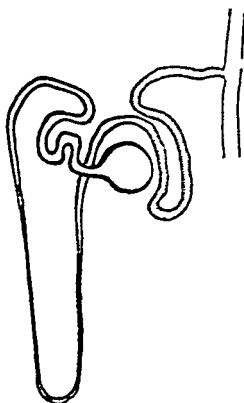
In the inner zone of the renal cortex were to be seen in every section many characteristic loops of Henle, mostly of a brilliant blue, but some violet, and a few red. Adjoining these portions bits of tubules were often made out from just above or just below the loops of Henle in which a transition from blue through violet to red or *vice versa* could be seen. The above mentioned unidentifiable bits of tubules containing the dye in violet form may be considered to have come from regions lying a little way above or below the loops of Henle.

In the outer cortical region convoluted tubules could be seen the cells of which all contained the dye in red granules. In no instances were the striations, characteristic of proximal convoluted tubules, present among them. Very often these "red tubules" emptied directly into the collecting tubes, in which case there was, of course, no doubt that we were dealing with distal convoluted tubules. In striking contrast, the proximal portions of the convoluted tubules, when definitely identified, either by their striations or when emerging from a glomerulus, contained the dye in the blue form. Never was any dye to be seen in the cells of the



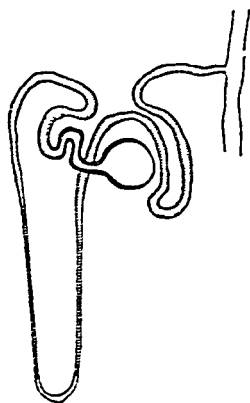
Neutral urine

TEXT-FIG. 1.



Alkaline urine

TEXT-FIG. 2.



Acid urine

TEXT-FIG. 3.

Text-fig. 1 shows diagrammatically the renal color pattern following intraperitoneal injections of erythrolitmin in rats secreting neutral urine. Text-figs. 2 and 3 show the changes in color pattern associated with the secretion of alkaline and acid urine, respectively, described in the text. The region of blue erythrolitmin is indicated by solid black, that of violet by dots, and of red by the short lines.

collecting tubules and none was ever recognizable in the glomeruli or tubular lumena.

Sometimes all of the findings described could be made out in a single preparation. From the examination of scores of fresh sections, from many animals, we have prepared Text-fig. 1, which shows diagrammatically the state of affairs in the kidney of a rat maintained on an ordinary diet of barley, oats, and bread and milk, and excreting a very slightly acid or a neutral urine.

In brief, then, during the excretion of neutral or very slightly acid urine, the granules of the lining cells of the glomeruli as well as those

of the cells of the derivative tubules, at times as far along as the loops of Henle, are alkaline to erythrolitmin. Further on, in a short segment of tubule the cell granules show the violet color of neutrality, while further yet, in the cells in the ascending limb and in the distal convoluted tubules, the granules are acid in reaction (Text-fig. 1). There is no dye anywhere to be seen in the lumina of the tubules and none within the cells of the collecting system.

The Effect of Diuresis on the Staining with Erythrolitmin.

A number of rats were provided for several days with nothing but a 10 per cent watery solution of cane sugar which they drank in great quantities. During this period erythrolitmin injections were given as in the instances described above. A series of control animals fed on a diet of bread and milk, barley and oats and injected with similar doses of erythrolitmin, were employed for comparison. The urine secreted by the sugar-fed animals appeared slightly more alkaline to brom cresol purple and phenol red than did that of the controls and was from two to four times as abundant. In the kidneys of individuals killed at the height of the diuresis, sections of the renal tissue, viewed in the gross, appeared to contain far more blue dye. Microscopically, fewer unstained areas were seen and the loops of Henle were oftener of a bright blue, though the cells of the distal convoluted tubules were still always red. The point of change from the blue to the red reaction was apparently situated further down the tubule.

The experiments suggested that with an increase in the quantity of the urine and a change to slightly greater alkalinity, more, relatively speaking, of the litmus was present in the alkaline form.

The Kidneys during the Excretion of Alkaline Urine.

Animals to which sodium bicarbonate had been given were now studied.

Rats weighing from 75 to 200 gm. were given by stomach tube, in the morning and again late in the afternoon of the same day, 5 cc. of a 10 per cent solution of sodium bicarbonate. A plentiful excretion of alkaline urine always resulted. On the 2 or 3 days following, during which the bicarbonate administration was continued, injections of a 2 per cent erythrolitmin solution were made as in the

normal control rats. There were either two or three injections, on successive or alternate days. After a final lapse of 48 to 72 hours, without discontinuance of the bicarbonate administration, the animals were killed and the kidneys examined microscopically. To vary the conditions some individuals, previously injected twice or thrice with erythrolitmin, were given the alkali once, then again on the following morning, and in the afternoon they were killed and studies of the kidneys were made. The findings were the same whichever the procedure.

In the gross the kidneys appeared deep purple-red as ordinarily. Microscopically the general color of the cortex was blue, but scattered areas of cells with brilliant red granules were to be found. As usual the glomeruli lay within blue crescents of endothelial cells packed with blue dye, and the capillary tufts were filled with blood of natural color. The cells of the proximal convoluted tubules leading away from the glomeruli were crowded with granules of a dense deep blue. Nearly all the tubular loops which could be identified as descending limbs of the loops of Henle were deep blue and so too with the ascending limbs for some distance. In the cortex, however, many red tubules were seen, the cells thereof containing in no instance the striations of proximal convoluted tubules. A connection of these red tubules with the collecting tubes could frequently be made out and hence they must have been portions of the distal convoluted tubules. But segments of them,—not adjacent to the collecting tubes but lying further up,—were blue or violet, a phenomenon never observed in the animals secreting neutral urine. The collecting tubules themselves were unstained.

A change in the relative reaction within the tubule cells as evidenced by the distribution of the litmus colors had evidently taken place in these animals. The blue coloration extended much further down, being seen in the granules of the cells of the ascending limb of the loop of Henle and in an adjoining portion of the distal convoluted tubules. In every case the terminal portions of these latter where they joined to the collecting tubules were red with the indicator, as under normal conditions, and this despite the fact that a markedly alkaline urine was being secreted.

The findings described above have been gathered from an examination of many sections from many animals, though occasionally a single specimen showed them all. By and large they demonstrate clearly that, during the secretion of alkaline urine, the reaction within the cells of the kidney cortex is markedly altered. The essential difference from the normal lies in an extension of the blue color along the tubular path, in some instances almost as far as the collecting tubules. Text-fig. 2 shows this diagrammatically.

Appearance of the Kidneys during the Secretion of Acid Urine.

Rats injected with erythrolitmin were caused to secrete acid urine. The kidneys showed changes the opposite of those occurring when the urine had been rendered alkaline.

The secretion of acid urine was brought about in rats by the administration of a 10 per cent aqueous solution of sodium acid phosphate through a stomach tube. At the same time control animals were fed ordinary food and other rats were fed alkali for the experiment already described. Rats of like age and weight were chosen for the procedures. Half of those given sodium acid phosphate received two doses in a single day and 24 hours later the first of the injections of erythrolitmin. Either two or three injections of the dye were made, on alternate or successive days, and the examination of the kidneys was carried out 48 hours after the last injection. Throughout the period of preparation, 5 to 8 days in all, the feedings of sodium acid phosphate were continued; and the animals' urine was regularly acid during the period. The other half of the rats were fed acid solution for the first time on the day after the last of several erythrolitmin injections, and again on the following morning. 6 hours later the kidney examination was made, at a time when acid urine was being formed. In both groups the findings were the same.

In the gross the kidneys appeared reddish purple. Microscopically the sections appeared almost entirely a bright red in contrast to the spotty red and blue of the controls. The outer areas of the cortex were red, with only here and there tubules containing the dye in blue form. The tubules of the inner cortical area were all red, including the loops of Henle. But it was striking that even in these kidneys the glomeruli lay cupped in blue crescents just as in the controls, the endothelial lining cells containing erythrolitmin only in the blue form. Wherever a tubule could be found leading off from the tuft the cells near the glomerulus likewise contained blue granules. Text-fig. 3 shows diagrammatically the findings in these experiments. The cells of the first portions of the proximal convoluted tubules contained the dye in the blue form only, but the changes from blue to violet and then to red occurred close to the glomerulus, considerably nearer than in normal animals, and far nearer than in the kidneys of the rats fed alkali. Frequently the shift in color of the intracellular granules was seen to occur at a point not more than 100 or 200 μ from the exit of the tubule from the tuft. In numerous instances the eye could trace the entire color sequence within a relatively short extent of tubule. Many individual cells in the region of color change where the granules were preponderantly violet contained both bright blue and brilliant red granules lying together in the cytoplasm. Distal to the region of change all of the intracellular granules were red. The few blue areas in the cortex could always be identified as segments of the proximal convoluted tubules from next the glomerulus. Cells containing dye in the blue form were never found further along. Brilliant

red areas were frequent that consisted of proximal convoluted tubules. No blue loops of Henle were seen; all were red. As in the other sections the collecting tubules were quite without color.

To sum up the alteration in the reaction within the tubule cells, as evidenced by the litmus change to red, affected the proximal convoluted tubules throughout almost their whole length and the entire loop of Henle. The structures mentioned now showed acidity instead of the alkalinity which is normal to them.

Constancy of the Findings.

The phenomena described occurred with but very little individual variation. Certain of them were constant. No matter what the reaction of the urine the granules of the cells lining the glomerulus and the adjoining portion of the proximal convoluted tubules were blue. And the intracellular granules of the further portion of the distal convoluted tubules were always red. Erythrolitmin was stored throughout the system from glomerular tuft to collecting tubule but not in either of these structures. In normal animals secreting a neutral or slightly acid urine the transition from red granules to blue, the zone that is to say where the granules are predominantly violet, is situated in the beginning of the ascending limb of the loop of Henle.

The relative reaction of kidney cells and their changes under functional conditions were studied in some other species besides the rat. Mice, guinea pigs, and rabbits were given intraperitoneal injections of erythrolitmin in dosages similar to those used in the rat experiments, that is to say $\frac{1}{4}$ to $\frac{1}{2}$ mg. of the dye per gm. of body weight. The secretion of acid and alkaline urines was induced in the way already described, by feedings of sodium acid phosphate or sodium bicarbonate; and the state of the kidneys was compared with that of normal animals injected with erythrolitmin. The findings were altogether similar to those in the rat, so they need not be enlarged upon.

Constancy of the Findings Following Massive Doses of Acid and Alkali.

Acid Administration.—A remarkable feature in the experiments just described was the regular occurrence of erythrolitmin in the blue form

TABLE I.

Rat No.	Body weight gm.	Daily administrations of HCl (concentration and amount)	Character of secretion of urine	Urine pH	Interval between last acid dosage and autopsy hrs.	Color of erythroblasts in glomerular lining cells
1	142	1 of N/10, 10 cc.	Copious secretion	6.0	16	Blue
2	150	4 of N/10 @ 10 cc.	Copious secretion	5.6	16	Blue
3	122	2 of N/10 @ 10 cc.	Few drops only on 2nd day	6.0	5	Blue
4	128	1 of N/6, 5 cc.	Copious secretion 6 hrs. later	5.9	6	Blue
5	125	1 of N/3, 5 cc., followed by water, 9 cc., in 1 hr.	Scanty secretion 2 hrs. later	5.4	2	Many blue
6	135	1 of N/4, 9 cc.	Scanty secretion 6 hrs. later	5.5	6	Many blue
7	99	1 of N/4, 10 cc.	Very scanty secretion 5 hrs. later	5.9	5	Many blue
8	104	" "		to		Change from blue to red in proximal convo- luted tubules very close to glomerulus
9	111	" "		6.2		
10	106	1 of N/4, 10 cc.	Hematuria 2 hrs. later. Sup- pression 5 hrs. later		5	Very few bluish purple
11	104	2 of N/10 @ 10 cc.	1st day copious secretion 2nd day suppression	5.5 to 6.0	5	Mostly purple A few red
12	129	2 of N/10 @ 10 cc.	1st day copious secretion 2nd day suppression	5.5	5	Few blue In proximal convoluted tubules cells near glomerulus purple to red Many purple Red in two glomeruli

13	120	2 of s/10 @ 10 cc.	1st day copious secretion 2nd day suppression Last day suppression	5.0	5	None really blue None blue	Several red-lavender Many red
14	110	3 of s/10 @ 10 cc. 1 of s/2, 2 cc.	Last day suppression		16	None blue	Many red
15	151	3 of s/10 @ 10 cc., a 2 day interval, then 1 of s/2, 2 cc.	Last day suppression		7	None blue	Some red
16	101	of s/4, 10 cc. 1 of s/3, 10 cc., followed in 1 hr. by 8 cc. water	2 hrs. later hematuria 6 hrs later suppression		6	Few blue	Some red
17	109	1 of s/3, 10 cc., followed in 1 hr. by 8 cc. water	2 hrs. later suppression 4 hrs. later suppression		4	None blue	None red
18	122	1 of s/2, 10 cc., followed in 1 hr. by 8 cc. water	2 hrs. later hematuria 5 hrs. later suppression		5	Few blue	Some red
19	168	1 of s/1, 14 cc.	1 1/2 hrs. later hematurin 3 hrs. later suppression		3	Few blue	A few red

within the cells lining the glomerulus and the adjoining portion of the proximal convoluted tubules, no matter what the reaction of the urine that was being secreted. Litmus segregated within healthy cells elsewhere throughout the body is regularly in the acid form, red that is to say. It was deemed worth while to test whether the blue of the glomerular litmus is changed to red by massive doses of acid.

For the purpose, rats weighing from 99 to 168 gm. were given dilute HCl by stomach tube in varying concentrations and amounts, as outlined in Table I. As a rule, several administrations of HCl were employed at 24 hour intervals, or a single dose in higher concentration. The animals were kept in special cages for the collection of urine under oil. Fresh bladder urine unmixed with feces was obtained for pH estimation with indicators by holding a closed cone over the animal's nose for a moment, thus inducing a struggle during which urine was voided. In all other respects the technique was that already described.

Table I summarizes the result of this experiment. The animals receiving the smaller quantities of acid (Nos. 1 to 9, inclusive) continued to secrete urine. In the kidneys of these rats erythrolitmin, present in the lining cells of the glomerulus and epithelial cells of the first portion of the proximal convoluted tubules, was still always blue. In both of these regions it frequently appeared bluish purple, but never frankly red. The color change of the dye in the cells of the proximal convoluted tubules took place much closer to the glomeruli than in the earlier experiments, often less than the distance of one glomerular diameter away from the exit of the tubule from the tuft.

Suppression of urine, usually preceded by hematuria, followed the large quantities of acid given to Animals 10 to 19, inclusive (Table I). In contrast to the findings of the foregoing experiments, erythrolitmin appeared at times in the red form within the glomerular lining cells and those of the first portion of the proximal convoluted tubules. But only after urinary suppression could this be demonstrated. Whenever secretion continued (Rats 1 to 9 inclusive), the dye in the locations named remained either blue or a deep bluish purple. A comparison of the findings of Rats 7, 8, and 9 with those of 10 shows this point well, for all these animals received similar amounts of acid but with differing results. So, too, does a comparison of the findings of 3 with those of 11, 12, and 13.

The effect of massive doses of acid may also be evoked by means of an "acid-forming" diet. Feedings of CaCl_2 have been shown by Haldane, Hill, and Luck (28) to have the effect of administrations of HCl; for the calcium moiety is *not* retained, while the chlorine is. According to Gamble, Ross, and Tisdall (29), 40 per cent of the chlorine given in this way behaves in the body like HCl. As Addis, MacKay, and MacKay have pointed out (30), a rat eating 10 gm. of diet containing 4 per cent of CaCl_2 should receive the equivalent of 14.4 cc. N/10 HCl.

We therefore maintained rats for 10 days on a diet of bread and milk to each 100 gm. of which 4 to 6 gm. of CaCl_2 were added. Eight of the animals were

injected with erythrolitmin on 2 alternate days and killed 48 hours after the last injection. The special diet was continued to the end. Urine was secreted plentifully and of a pH usually well below 6.0. Upon examination of the kidneys the dye still appeared blue in the glomerular lining cells and the first portion of the proximal convoluted tubules.

Alkali Administration.—Massive doses of alkali were given to rats stained with erythrolitmin, to see whether in this way the color of the dye present in the lining cells of the final portions of the distal convoluted tubules could be influenced. Even after the feedings of sodium bicarbonate outlined earlier in this paper it had been constantly in the red form.

Stained rats were given by stomach tube 4 cc. or 2 cc. of N/1 Na_2CO_3 solution per 100 gm. of body weight. The larger doses led to rapid suppression of urine, the smaller often to a similar result after 48 hours. The urine when obtained was usually of a pH about 8.2, as determined by the indicators cresol red and thymol blue. Examinations of the kidney were made, in many instances, before this result had been attained, and in others afterwards. We were never at any time able to demonstrate the dye in the blue form within the lining cells of the final portion of the distal convoluted tubules where these were actually observed emptying into the collecting tubules. The dye was frequently of a reddish lavender but never frankly blue. In all other situations throughout the tubular epithelium, it was intensely blue.

It is evident that massive doses of acid fail to change from blue to red the erythrolitmin segregated in the cells lining the glomeruli and the first portion of the proximal convoluted tubules. As long as the kidney continues to secrete urine these cells maintain a relative alkalinity, despite the strong acidity of the urine. Only in the event of urinary suppression is there some slight shift in the direction of acidity.

After massive doses of alkali, sufficient sometimes to cause urinary suppression, the erythrolitmin within the cells lining the final portion of the distal convoluted tubules remains red.

Results after Intravenous Injections.

To complete our observations some intravenous injections of erythrolitmin were made. The method has already been used by Stieglitz (7) who employed azolitmin, neutral red, and sodium alizarin upon rabbits and dogs.

Rats of approximately 100 gm. weight were injected intravenously with 1 or 2 cc. of a 1 per cent solution of erythrolitmin at a pH of approximately 7.0. The kidneys were extirpated and sectioned after intervals of 30 minutes, 1 hour, 2, 5, 6, 10, and 18 hours. The animals tolerated the dye poorly and in many instances became moribund a few minutes after the injection.

The findings were by no means as clear-cut as after intraperitoneal injection of the indicator since the kidneys never became well stained.

Within a few seconds after intravenous injection of erythrolitmin the urine contained it in high concentration whereas the kidney tissue showed not the slightest traces. When half an hour had elapsed the glomeruli appeared faintly bluish regardless of the reaction of the urine. The color seemed to be due to the presence of the dye in the blood within the tufts. When 2 hours had elapsed some of the tubular epithelial cells of kidney sections showed a very faint diffuse blue tint. No definite coloration of intracellular granules could be made out, and the staining was so slight that it appeared merely as a blue haze over the cells. 6 to 8 hours after an injection of the dye, colored granules could be found here and there in the tubule cells. Both red and blue granules were to be found, indiscriminately located. In this connection it may be recalled that litmus is often taken up by cells in fine blue particles and only secondarily converted to red (9) as further that when a cell containing segregated litmus is damaged or has begun to degenerate it turns from red through violet to blue (9). The coloration of the kidney cells was so poor, as compared with that obtained after intraperitoneal injections, that no conclusions as concern kidney function could be drawn from the instances mentioned.

With a further increase to 24 or 48 hours in the time interval between the injection of erythrolitmin and the examination of the kidney, the appearance of the organ came to resemble closely that following intraperitoneal injections. Nothing, then, had been gained by the intravenous administration of the indicator.

We were unable to secure satisfactory staining of the rat's kidney after intravenous injections of azolitmin (Grübler), with our own preparation of azolitmin or with sodium alizarin VI, dyes with which Stieglitz (7) reported success in dogs. Exitus frequently occurred soon after injection of the dyes when given in quantities too small to stain the kidneys.

It has been reported by Stieglitz that, after the intravenous injection of azolitmin, sodium alizarin, and neutral red, the reaction of the kidney cortex is apparently the opposite of that of the urine secreted at the time. In view of our failure to confirm this finding with the aid of the dyes just mentioned it seemed necessary to repeat Stieglitz's experiments with neutral red.

Solutions of neutral red in physiologic saline when given intravenously stained the kidney well in dogs and rabbits within a few minutes. The tubule cells took up the dye in both a diffuse and granular form and it appeared promptly in the urine. However, under these circumstances there was no marked differential staining, as with erythrolitmin. The kidney was always of an almost uniform hue, which hue depended largely, as will be shown, upon the dosage of the dye and the time interval between its injection and the examination of the organ. In passing it should be said that the yellow, alkaline form of neutral red can easily be confused with the natural yellowish color of thin sections of kidney tissue.

Six dogs and 16 rabbits were induced to secrete alkaline or acid urine, as desired, through the oral administration of sodium bicarbonate or sodium acid phosphate, or by the intravenous injection of these substances. After the bladder urine obtained by catheterization had become definitely alkaline or acid, intravenous injections of neutral red (Grübler) were given, in 1 per cent or 0.5 per cent solution in physiological saline. Various doses were used and the kidney examined after various intervals of time. Specimen protocols follow:
Normal dog, 8½ kilos.

10. 50 a.m. Ether anesthesia induced; the bladder urine is distinctly acid to brom cresol purple and neutral red. 10.52. Catheterization; urine specimen acid as before; catheter bound in.

10.55. *30 cc. of 1 per cent solution of neutral red (Grübler) in 0.9 per cent NaCl solution injected intravenously.* The animal became rose red at once. 11.01. The dye appeared in the urine, in the red form. 11.25. The right kidney was removed through a posterior incision. In the gross it appeared deep red. In sections, cut with a Valentine knife and mounted between mica slides, the tubular epithelial cells were colored red, and many contained the dye in granular form. The general staining of the kidney, however, was slight.

11.30. *Intravenous injection of 5 per cent solution of sodium bicarbonate, 150 cc.* 11.33. The urine became orange. 11.40. The urine was clear and dark yellow; it turned red upon the addition of acid. 12.00 m. The left kidney was removed. In the gross the whole organ appeared yellowish. Sections, made with the Valentine knife and mounted as described above, appeared yellow. The tubular epithelial cells were stained diffusely yellow.

In this experiment the kidney removed after the animal had begun to secrete alkaline urine appeared definitely more alkaline than the organ examined at a time when the urine was acid.

In another experiment after an intravenous injection of 5 per cent sodium bicarbonate with consequent elimination of alkaline urine, a larger intravenous injection of 1 per cent neutral red was given, 45 cc. to a 9 kilo dog, and the kidneys were removed 12 minutes later instead of after half an hour, as in the experiment above. Microscopically the epithelium of the renal tubules appeared red although the urine secreted was yellow, a finding the opposite of that just described, but in agreement with the experiments of Stieglitz (7). The test was repeated on

another 9 kilo animal with the same dosage of dye but one of the kidneys was removed half an hour after an alkaline reaction of the urine had been established, instead of after the lapse of but 12 minutes as in the previous experiment. The second kidney was removed 30 minutes after an acid reaction of the urine had been established. With this greater interval of time between the injection of dye and the examination the color of the kidney cortex was found similar to that of the urine secreted. The protocol follows:

Normal dog, 9 kilos. All urine specimens obtained by catheter.

10.45. a.m. Ether anesthesia. 10.50. Urine clear and strongly acid to neutral red, erythrolitmin, and brom cresol purple.

10.58. *Intravenous injection of sodium bicarbonate, 5 per cent solution, 75 cc.*

11.08. Intravenous injection of neutral red, 1 per cent, 40 cc. 11.14 (6 minutes later). Urine appearing red from the catheter,—still acid. Intravenous injection of sodium bicarbonate repeated. 11.17. Urine bright orange, that is to say definitely alkaline. 11.47. Urine has been alkaline for $\frac{1}{2}$ hour. Right kidney removed. Histological study made at once (see below).

11.49. *Intravenous injection of sodium acid phosphate solution, 5 per cent, 70 cc.*

11.51. Urine now reddish rose colored. 11.53. Injection of sodium acid phosphate repeated. 11.55. Urine bright rose red, strongly acid. 12.21 p.m. Urine has been acid for $\frac{1}{2}$ hour; left kidney removed. Histological study made at once. Animal killed with chloroform.

The kidneys were removed, in each instance 30 minutes after the urine had been rendered acid or alkaline, respectively. When the animal was passing yellow alkaline urine the gross appearance of the kidney was a reddish yellow. Microscopically, sections mounted on mica slides and ringed with paraffin showed a moderate, diffuse, and granular staining of the cortex. The dye in the tubule cells was mostly in the yellow form, but some in the red. The predominating color, though, was yellow, like that of the urine.

When acid urine was being secreted the kidney appeared bright red in the gross. Nearly all the dye in the epithelium of the tubules was present in the red form, with here and there a little yellow coloration. Again the predominating color of the cortex was like that of the urine.

Such experiments were many times repeated, with different doses of neutral red. The dye was given in the red form, but was brought close to the neutral point with sodium bicarbonate. It cannot be injected in the yellow form because of the resulting precipitation of the dye in alkaline reaction. In general, when large doses of the dye were given and examination of the kidney made shortly afterwards, the tubule cells contained much of it in the red form, regardless of the reaction of the urine. When half an hour or more was allowed to elapse between the injection of the dye and examination of the kidney,

and when the dosage of dye was not too great, the majority of epithelial cells manifested the same color as did the urine.

Similar experiments upon rabbits gave findings of like sort. When large doses of neutral red were employed and the examination made soon thereafter, the kidney cortex, in general, appeared red, no matter what the reaction of the urine. Lengthening the interval between injections of the dye and examinations of the kidney gave time for the organ to convert most of it in the tissues to the form in which it appeared in the urine.

The protocol which follows contains the findings in four rabbits, and shows well the influence of dosage, and time interval, upon the coloration of the kidney cells with neutral red.

The treatment of the animals may be tabulated as follows:

Rabbit	a	b	c	d
Weight ..	650 gm.	655 gm.	647 gm.	653 gm.
Intravenous injection of 5 per cent sodium bicarbonate	8 cc.	8 cc.	8 cc.	8 cc.
5 min. later.				
Intravenous injection of 1 per cent neutral red	12 cc.	2 cc.	12 cc.	2 cc.
Kidney examination	5 min. later	5 min. later	90 min. later	90 min. later

After the bicarbonate injection the urine of all the animals was strongly alkaline and contained much dye in the yellow form.

Rabbit a—In the gross the kidney appeared dark red. Section showed the tubule cells containing much red dye, in granular as well as diffuse form. The cortex was predominately red.

Rabbit b—The kidney appeared red in the gross, but lighter in color than that of Rabbit *a*. Sections were yellowish red. The tubule cells contained dye in red granules. The general appearance of the kidney of *a* was more acid than that of *b*. 5/50 HCl run under the cover-glasses turned the sections as a whole a deeper red, showing that some of the dye was present in the yellow form.

Rabbit c—The kidney appeared a mottled reddish yellow. The sections were red in general and deeply stained. Many tubule cells held the dye in red granular form, while in other tubules the cells contained yellow granules. The glomeruli appeared as distinct yellow crescents. The urine was a deep yellow. The

bladder, however, contained yellow urine with clot-like masses of dye in the red form. When the contents were mixed the prevailing hue was yellow.

Rabbit d.—In the gross the kidney appeared yellow. Sections were also yellow. There was more dye present in the tubule cells than in those of Rabbit *b* which received the same dose of the indicator, and less than in Rabbits *a* and *c*. Practically all of it was present in the alkaline form.

From the results of these and other like experiments certain facts became plain. When the dose of neutral red was small the tubular epithelium of the kidney usually appeared of the same color as the urine secreted, though in a few instances it had the opposite color. When the dose was large enough to stain the tissue well much of the indicator within the cells manifested the color it had when injected, regardless of the reaction of the urine. When the time interval between the injection and the examination of the kidney was short, that is to say half an hour or less, the cells might or might not have the same color as the urine. If the time interval was lengthened, the color of the majority of the kidney cells was that of the urine being secreted. Apparently the lengthening of the interval between the intravenous injection of a large amount of dye and the histological examination gave time for its conversion in the stained tissues to the form in which it appeared in the urine. If the time interval after injection was short, it seemed largely a matter of chance how the kidney was stained owing to the great concentration and overwhelming amount of material suddenly brought to it.

Altogether it is obvious that the results of the intravenous method of administration of indicator dyes for renal staining are untrustworthy, unless examination of the organ can be postponed until the cells can deal with the material coming so suddenly to them. In practice many indicator dyes are toxic, when administered in this way, and the animals frequently die before sufficient time for a proper staining of the tissues has elapsed.

DISCUSSION.

Erythrolitmin is far from being a perfect indicator (24). In view of this fact how much is one justified in inferring from the present work? The color of most indicators varies with their concentration

and erythrolitmin is no exception to the rule. There is then a definite, though slight, increase in the red in higher concentrations; and since erythrolitmin would appear to be often very highly concentrated when segregated within the kidney tubule cells, the possibility of an interpretative error from this source may be great. The dye is subject to large salt and protein errors as well, but these influence it in the opposite direction. Erythrolitmin in the presence of salt or protein at a given pH appears more blue than in solutions at the same pH without these ingredients (24).

Granting these facts erythrolitmin still has notable virtues as an indicator of tissue conditions. It persists within the tissues for months, eliciting no evident reaction and retaining its indicator characteristics. This is true of the dye segregated within renal epithelium. When a section of the stained kidney is placed in a buffer solution all the stained granules take the same color, namely one approximating that which would be expected of erythrolitmin at the pH of the buffer. In untreated specimens immediately after removal from the body many granules containing the dye in highly concentrated form are a deep blue. Since the optical error upon concentration is toward the red, it is evident that this factor plays no decisive part in the color.

Can the findings of the experiments outlined in the present paper be due to supravital changes? The constancy of the results under a given set of conditions and the equal constancy of the induced variations would seem to put this possibility out of the question. One might suppose that the knife in sweeping from cortex to medulla and carrying with it a mixture of blood, lymph, glomerular filtrate, and more or less completed urine would bring about an alteration in the granules of the cells with which it came in contact. So it must in those cells that are injured. Such an effect will not explain, however, the orderly arrangement of red and blue granules everywhere in the unexposed cells. These cells show no change in the litmus coloration for many minutes after removal from the body. The kidneys can be removed from living animals, sectioned, and observed, within 45 seconds to 1 minute. Renal cells can scarcely be regarded as dead in so short a time, much longer interruptions of the circulation being withstood (31).

Can one suppose the variations in the reaction of the granules in the living cells of the renal tubules to be a mere passive reflection of the reaction of the fluid passing along the tubules? Such a view is incompatible with what is known of cell integrity. Litmus segregated in granular form by phagocytes does not take the color appropriate to the hydrogen ion concentration of the fluid surrounding these cells unless they are crushed or otherwise injured (9).

All things considered, one is forced to the conclusion that the color patterns seen in kidneys stained with erythrolitmin are the expression of functional activities. But how these activities exert their influence on the reaction of intracellular granules is a problem of great complexity.

The fact has already been emphasized that the granules within the epithelium of the glomerulus and the adjoining portion of the convoluted tubules are regularly blue, no matter what the reaction of the urine. Were it not for the cell integrity just mentioned the finding might seem explicable on the basis that the renal filtrate as derived from the blood and bathing these cells is ordinarily alkaline. Everywhere else though throughout the body litmus-containing elements are bathed by alkaline fluids yet the color of the indicator within them is red, indicative of acidity. Why should there be this peculiar exception in the case of this region of the kidney? And what is one to think of the shifting in the renal color pattern that can be brought about by administering substances to the animal which cause the reaction of the urine to shift to the alkaline or acid side. The tubule cells adjoining Henle's loop constitute, as it were, a transitional zone of color change. In them, erythrolitmin granules are violet, under normal circumstances, or sometimes red and blue in the same cell. During the secretion of acid urine the cells of this region contain the dye in the red form. One might suppose the red of the granules in this region to be of no greater significance than that met elsewhere in the body were it not for the fact that the red can be altered to blue by altering the functional conditions, as during the secretion of alkaline urine. One can only suppose these phenomena to be an indirect expression of functional activities on the part of the renal cells—cells through which material is passing into or perhaps out of the blood. Only in the case of the lining of the distal convoluted tubules where these join the collecting

system is another explanation possible. Through thick and thin, so to speak, the litmus within such cells remains in the red form, the form in which it is encountered in healthy, non-secreting cells elsewhere in the body. Here there would seem to be no reason to invoke a functional passage of material through the cytoplasm in order to explain the findings. Since the reaction within these cells cannot be rendered alkaline by any of the experimental means as yet attempted, it is conceivable that it is held fixed, as is certainly not the case higher up. Yet since these cells differ in no wise morphologically from those immediately adjacent to them in the same tubule, and do differ sharply from the elements of the collecting tubules upon which they abut, it seems more likely that they are protected by a fortunate situation from those functional stresses which in our experiments led to a change in the reaction of the granules further up.

Granted that the renal color pattern and its changes are the expression of functional activities, what is the nature of these latter? Do the cells lining the tubules resorb fluid only, or secrete materials into the tubular lumen as well? Unfortunately the findings here described would appear to admit of both possibilities. One can say at the moment only that the reaction within the cells of the renal tubules varies from region to region in a way which shows it to be intimately connected with the secretory activities of the organ. In some regions it is profoundly altered by changes in these activities.

Stieglitz' (7) conclusion that the reaction of the kidney cortex is the reverse of that of the urine being secreted at the time is based on observations of the cortex color following intravenous injections of indicators. Our repetition of some of his experiments has shown that the color of the kidney cortex shortly after such injections is influenced chiefly by factors not considered by Stieglitz, namely the dosage of the dye and the variations in the time interval between its injection and the examination of the organ. Large amounts of indicator dye coming suddenly to the kidney from the blood may be found, a few minutes later, unchanged in color within the cortex cells regardless of the reaction of the urine. The kidney overwhelmed, so to speak, with dye requires an appreciable time to act upon all the indicator substance present. A heavy dose of neutral red, in the acid form, injected intravenously into animals secreting alkaline urine, will appear in the

kidney cortex a few minutes later still in its red, acid form, indicating a reaction the reverse of that of the urine being secreted at the time. Later the dye in the cortex will be in the yellow, alkaline form. Small doses of neutral red intravenously injected in the acid form into animals secreting alkaline urine are of course converted to the alkaline form in the kidney sooner than are large doses. When acid urine is being secreted, neutral red injected in the acid form remains unchanged regardless of the dosage given.

Such findings clearly show how the employment of indicators by intravenous injection may lead to erroneous conclusions concerning the relation between the reaction of the kidney cortex and that of the urine secreted. They show further that the examination should be postponed until the cortex cells have had ample time to deal with the dye coming to them. Under these conditions one finds the reaction of the kidney cortex to be, by and large, the same as that of the urine under secretion.

SUMMARY.

The tubules of the mammalian kidney, vitally stained with erythrolitmin, show a significant color pattern, the cells of certain regions appearing bright blue and others brilliant red. The dye is segregated within the cytoplasm, staining fine granules diffusely.

Under normal circumstances of renal function erythrolitmin is stored in the lining cells of the glomerulus and the epithelial cells of the proximal convoluted tubules in the blue, alkaline form. In the cells of the final portion of the distal convoluted tubules the dye is red. Between these regions a narrow transitional zone can be found, at times above, at times below the loop of Henle, in which the erythrolitmin-stained granules in the tubule cells are violet, or red and blue within the same cell.

Alterations in the relative reaction of certain regions of the tubules, as disclosed by the color of the dye within the cells, can be induced by means that alter the reaction of the urine. When acid urine is being secreted there is a change from relative alkalinity to acidity high up in the tubular canal, in the proximal convoluted tubules. During the secretion of markedly alkaline urine the intracellular granules appear blue most of the way down the tubular canal, even to the first portion

of the distal convoluted tubules. When the urine is neutral the cells above the descending limb of the loop of Henle are alkaline to erythrolitmin, and those below this point appear acid.

The granules within the endothelial cells of the glomerulus and the epithelial cells of the first portion of the proximal convoluted tubules are always alkaline to erythrolitmin; while those within the cells of the final portion of the distal convoluted tubules are always acid regardless of the reaction of the urine. Only after complete suppression of urine as result of massive doses of acid do granules of the sort first mentioned manifest the color indicative of acidity.

The interpretation of the findings waits upon further work.

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ETIOLOGY OF OROYA FEVER.

XII. INFLUENCE OF MALARIAL INFECTION (*PLASMODIUM INUI*?), SPLENECTOMY, OR BOTH, UPON EXPERIMENTAL CARRION'S DISEASE IN MONKEYS.

By HIDEYO NOGUCHI, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

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Mixed infections with *Bartonella bacilliformis* and plasmodia are not uncommon in regions where both malaria and Carrion's disease (verruca peruana, Oroya fever) are endemic. Both parasites induce swelling of the spleen, and both invade the red blood cells. It is conceivable that the preexistence of malaria might have an unfavorable influence upon a subsequent infection with *Bartonella bacilliformis*, as a result of impairment of the defensive powers of spleen and blood by the plasmodia, or that the introduction of plasmodia during the course of a dormant or partially controlled verruca infection might result in renewed invasion of the blood and lymphatic system by *Bartonella bacilliformis*. The abrupt development of malignant Oroya fever in the course of apparently benign verruca would be explicable on this latter hypothesis.

The opportunity to obtain definite experimental data on the effect of malaria infection on the course of experimental verruca presented itself when malaria parasites were found in the blood of a monkey (*Macacus cynomolgus*) which had been splenectomized in connection with an experiment to determine what influence the operation would have upon the susceptibility of monkeys to *Bartonella bacilliformis*.

Protocol 1.—*Macacus cynomolgus* 1-M, splenectomized on Dec. 1, 1926, to serve as control for *M. cynomolgus* 2-S, which was splenectomized the same day and inoculated with *Bartonella bacilliformis*. Examination of the blood of Monkey 1-M was made daily in order that the appearance of any *Bartonella*-like intracor-

puscular elements (such as appear in splenectomized rats¹) might be detected. These elements were not found, but the examinations revealed plasmodia² on Dec. 6, 9, 10, 11, and 16, 1926, and the parasites were still present at the time of death on Mar. 10, 1927. No fever was detected in this animal at any time. The monkey had shown no signs of illness during several months of captivity previous to the operation. It would appear that it was a chronic malarial parasite carrier, a condition already established for man. Death may have been due to aggravation of the malarial infection through splenectomy.

Preexisting or Concomitant Malarial Infection and Verruga.

A *Macacus rhesus* intravenously inoculated with the blood of *Macacus cynomolgus* 1-M on December 10 soon developed fever, and the malarial parasites were found in the blood. One month later verruga material was intradermally inoculated. In due course the cutaneous lesions appeared and attained moderate size. Blood taken on February 3, 1927, yielded cultures of *Bartonella bacilliformis*, but no severe form of blood invasion and anemia developed. The animal recovered from both infections within a few weeks.

Protocol 2.—*Macacus rhesus* 2-M, inoculated intravenously with 0.5 cc. of the blood of Monkey 1-M on the day when plasmodia were first noticed (Dec. 10, 1926). The febrile reaction began on Dec. 15, that is, 5 days after inoculation, and continued daily for 9 successive days, being high (105–106.2°F.) for 5 days and moderate (104.2–104.6°) for 4 days longer. The plasmodia were present on Dec. 16. The temperature was normal (about 102°) from Dec. 26 to Jan. 3 but rose to 104.6° on Jan. 4, 1927, and to 106° on Jan. 5. Plasmodia were found in the blood on Jan. 3. After Jan. 5 there was no more fever. On Jan. 10, 1927, cultures of *Bartonella bacilliformis* and a saline suspension of the nodule of *M. rhesus* 73 were intradermally inoculated. The signs of verruga were already evident on Jan. 19, and by Feb. 3 the nodules were of moderate size, and the blood had a culture titer of 1:100. The animal had no further paroxysm of malaria and recovered from both infections within a few weeks.

Another monkey was inoculated simultaneously with malaria and verruga material.

Protocol 3.—*Macacus rhesus* 3-M was inoculated on the same day as *M. rhesus* 2-M with 0.5 cc. of blood containing plasmodia and in addition received, both

¹ Mayer, M., *Arch. Schiffs- u. Tropenhyg.*, 1921, xxv, 150. Mayer, M., Borchardt, W., and Kikuth, W., *Klin. Woch.*, 1926, v, 559; *Arch. Schiffs- u. Tropenhyg.*, 1927, xxxi, 295. Noguchi, H., *J. Exp. Med.*, 1928, xlvii, 235.

² The parasite would appear to be *Plasmodium inui* (Wenyon, C. M., *Protozoology*, New York, 1926, ii, 968–973).

intravenously and intradermally, cultures of *Bartonella bacilliformis* and a suspension of verruga tissue. The animal's blood showed plasmodia on Dec. 16, 1926, 3 days before the onset of fever. The first paroxysm lasted 3 days (temperature 104.6°, 106.2°, 106.2°). The second rise of temperature (to 105.4°) was first detected on Dec. 27, temperatures not being taken daily during the Christmas holidays, and fever was still present on Dec. 28 (104°). Plasmodia were present in the blood on Jan. 3, and there was fever on Jan. 4, 5, and 6 (104°, 105.8°, 104°). Verruga nodules arose at the sites of intravenous inoculation into the saphenous veins (where several unsuccessful punctures had been made in the course of intravenous injection), but none at the sites of intradermal injections on the abdomen. Blood cultures made on Dec. 21, 1926, failed to yield cultures of *Bartonella bacilliformis*. The temperature of 106.2°F., recorded on that day, may have been due either to malaria or to verruga. A second injection of virulent verruga material intradermally on Jan. 10, 1927, resulted in only slight induration at the sites of inoculation, but the nodules on the back of the legs reached moderate size, and marked edema of the scrotum developed. The animal recovered.

The experiments outlined gave no evidence that the malarial infection in *Macacus rhesus* influenced unfavorably the result of infection with *Bartonella bacilliformis* when the plasmodia were introduced a month previous to inoculation with verruga or when both parasites were simultaneously inoculated.

Malarial Infection during Convalescence from Verruga.

The lesions resulting from experimental infection with *Bartonella bacilliformis* in monkeys usually heal within a few months, the microorganisms persisting longest in the spleen and lymph glands, where they can be demonstrated late in convalescence. It was of interest to determine whether, at this stage, the infection would again become active as a result of introduction of plasmodia.

As the protocols show, the inoculation of malaria parasites during convalescence had no influence whatever upon the course of recovery. In one instance the animal was reinoculated with virulent verruga material a month after the introduction of the plasmodia, but reinfection was not induced. The malarial infection apparently had not interfered with the development of immunity.

Protocol 4.—*Macacus rhesus* 4-M had had an infection with *Bartonella bacilliformis* during the autumn, but the skin lesions had almost healed. It was inoculated with 0.5 cc. of the malarial blood intravenously on Dec. 10, 1926. There

was fever lasting for 6 days, Dec. 22 to 27, 1926 (temperatures varying from 104.4° to 105°), and the blood was positive for plasmodia on Jan. 3, 1927. Blood culture was negative for *Bartonella bacilliformis* on Dec. 21, 1926, and the skin lesions had disappeared.

Macacus rhesus 5-M, also recovering from verruga, was inoculated with 0.5 cc. of malarial blood at the same time as the foregoing animal. Fever began on Dec. 16, 1926, and lasted 5 days (temperatures 104°-106.8°). Plasmodia were present on Dec. 16, 1926, and on Jan. 3, 1927. On Dec. 28, 1926, the temperature rose to 104.4° but fell to 103.6° the next day, and there were no later paroxysms. On Jan. 8, 1927, verruga material was inoculated intradermally. No skin lesions appeared at the old sites, and none developed at the sites of the new inoculations, and the blood remained negative for *Bartonella bacilliformis* by culture. Observation ended Jan. 29, 1927.

Effect of Splenectomy upon Verruga Peruana.

In connection with the problem of the nature of the intracorpuseular bodies known as *Bartonella muris*,¹ which appear in the red cells of rats following splenectomy, it became of interest to determine what effect splenectomy would have upon the course of experimental infection with *Bartonella bacilliformis*.

A monkey splenectomized on November 12, 1926, and inoculated soon afterwards with verruga material, developed the severe type of Carrion's disease (Oroya fever), with marked anemia, extensive edema, emaciation, and skin lesions. Even more violent reactions than the one in this animal (*Macacus rhesus* 1-S) are occasionally observed in *rhesus* monkeys as a result of inoculation with *Bartonella bacilliformis*,^{3,4} and hence the severity of the infection in this instance cannot be ascribed to the effect of splenectomy. Moreover, *M. cynomolgus* 2-S, which was subjected to splenectomy and simultaneously inoculated with nodular material from Monkey 1-S, showed a moderate reaction only. Although the blood titer was high (1:100,000), there were no other evidences of systemic infection, and the nodules were of medium size. The control monkey in this instance (*M. cynomolgus* 5-T) reacted more severely than the splenectomized animal, although the blood titer was only 1:10, as was that of a *rhesus* control.

³ Noguchi, H., *J. Exp. Med.*, 1927, xlv, 175.

⁴ Noguchi, H., *J. Exp. Med.*, 1926, xlv, 697.

Protocol 5.—*Macacus rhesus 1-S*, splenectomized Nov. 12, 1926, was inoculated 3 days later intravenously with 1 cc. of culture of *Bartonella bacilliformis* and 1 cc. of saline suspension of nodular tissue from *Macacus rhesus 2-T*.⁵ The same material was injected intradermally. A temperature of 104° to 104.6°F. was recorded on Nov. 11, 24, Dec. 10, 20, and 21, and on Jan. 15, 1927. Except on these 6 days the animal was afebrile. Blood cultures were positive on Nov. 24 (1:10,000), Dec. 8 (1:100,000), and Dec. 28 (1:100). The organisms were sufficiently numerous in the blood on Dec. 3 to be detected by examination of stained films. The nodules developed to moderately large size and persisted for about 2 months. From Dec. 11, 1926, to Jan. 3, 1927, there was general edema, especially marked in the scrotum, and a generalized nodular eruption.

Blood Counts.

	R.B.C.	Hemoglobin (Sahli) per cent
Nov. 12.....	5,456,000	90
Nov. 24.....	4,424,000	80
Dec. 27.....	3,080,000	50

The animal had completely recovered by Feb. 7, 1927.

M. cynomolgus 2-S, splenectomized Dec. 1, 1926, and inoculated intradermally with a culture of *Bartonella bacilliformis* and a saline suspension of nodular tissue from *M. rhesus 1-S*. Nodules of moderate size had developed by Jan. 14, 1927. Blood taken on Jan. 6, 1927, yielded cultures of *Bartonella bacilliformis* in a dilution of 1:100,000. The nodules had disappeared by Feb. 9, 1927.

*M. cynomolgus 5-T*⁵ was inoculated in the same way and with the same material as the foregoing animal but was not splenectomized. There was no fever at any time, but enormous cherry-red nodules developed on the eyebrows and abdominal wall, reaching their maximum growth on Dec. 27, 1926. The blood titer was 1:10 on Jan. 6, 1927, but blood culture was negative on Jan. 24.

Splenectomy did not induce relapse of experimental verruga infection in two animals which had recovered (*M. rhesus 40* and *41*⁵), nor did removal of the spleen render recovered animals susceptible to reinoculation with *Bartonella bacilliformis* (*M. rhesus 49, 50, 68*). Observation continued for a period of 1 month after inoculation of *Bartonella bacilliformis* into these animals failed to reveal any skin lesions, and blood cultures were uniformly negative throughout this period.

Effect of Splenectomy and Malarial Infection Together upon Verruga Peruana.

Two animals were subjected to splenectomy as well as to infection with plasmodia and *Bartonella bacilliformis*. One succumbed to the

⁵ Noguchi, H., *J. Exp. Med.*, in press.

malarial infection before the verruga lesions had had time to develop; the other had a moderately severe infection with *Bartonella bacilliformis* and while recovering died of tuberculosis.

Protocol 6.—*Macacus rhesus 6-M* was splenectomized and at the same time inoculated with 0.5 cc. of malarial blood on Dec. 10, 1926. The plasmodia were found in the blood on Dec. 16, and 4 days later the temperature rose and remained high for 3 successive days (104.2–106°). The second paroxysm began on Dec. 29, 1926, and lasted for 8 days (temperatures 104–106.6°). The plasmodia were very numerous on Jan. 3. The animal was inoculated with verruga material on Jan. 8, 1927, but succumbed to the malarial infection on Jan. 14, 1927, before the verruga lesions had had time to develop. *Bartonella bacilliformis* was not recovered in blood cultures.

Macacus rhesus 7-M was splenectomized on Dec. 10, 1926, and at the same time inoculated with 0.5 cc. of malarial blood and 0.5 cc. of verruga cultures and tissue, the latter intradermally as well as intravenously. The first paroxysm occurred on Dec. 20 and lasted 4 days (temperatures 104.2–106°), the second, beginning Jan. 3, lasted 5 days (temperatures 104.6–106.4°). Examinations for plasmodia were positive on Dec. 16, 1926 (+), and on Jan. 3, 1927 (+++). Blood taken on Dec. 21, 1926, yielded cultures of *Bartonella bacilliformis* in a dilution of 1:10,000. The animal died of tuberculosis on Jan. 10, 1927.

SUMMARY.

The experiments reported were designed to determine the influence of malarial infection (*Plasmodium inui?*), splenectomy, or both combined, upon the course and character of experimental infection with *Bartonella bacilliformis* in monkeys (*Macacus rhesus* and *M. cynomolgus*).

Blood withdrawn from a monkey showing spontaneous malarial infection was inoculated intravenously into monkeys (a) 1 month prior to inoculation with virulent verruga material, (b) simultaneously with the verruga material, and (c) during convalescence from verruga infection of moderate severity. All the monkeys contracted the malarial infection and suffered one to three paroxysms during a period of about a month. The verruga lesions appeared in the inoculated animals in due course, were of average size, remained for the usual length of time, and *Bartonella bacilliformis* was recovered in culture from blood which also contained the plasmodia. The lesions in the convalescent animals continued to heal at the normal rate, and blood cultures were negative for *Bartonella bacilliformis*, as is usual during

convalescence. One of the recovering animals was reinoculated with virulent verruga material a month after the injection of the malarial blood, but neither did new lesions arise nor old ones recur. The malarial infection, therefore, had no effect upon the course of verruga or upon the establishment of immunity to *Bartonella bacilliformis*, hence it would appear that malaria and verruga may coexist in the same individual without unfavorable effect of one disease upon the course of the other.

Similarly, splenectomy led to no appreciable aggravation of *Bartonella* infection. One monkey subjected to splenectomy and inoculated with verruga material shortly afterwards had an unusually severe reaction, but another, which was infected with material from the first and simultaneously splenectomized, reacted only moderately, while the non-splenectomized control showed a severer type of cutaneous infection. Even the combination of splenectomy and malarial infection did not appreciably aggravate the experimental verruga. Neither relapse of verruga nor reinfection with *Bartonella bacilliformis* was induced in convalescent or recovered monkeys as a result of splenectomy.

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PEYTON ROUS, M.D.

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EXPERIMENTS ON THE VISIBILITY OF THE POLYHEDRAL VIRUSES.

BY R. W. GLASER AND E. V. COWDRY.

(From the Department of Animal Pathology of The Rockefeller Institute for Medical Research, Princeton, N. J., and the Laboratories of The Rockefeller Institute for Medical Research, New York.)

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In recent years the conception of the existence of filterable viruses as excitants of disease has been considerably extended.¹ It is now generally recognized that these agents, whatever they may be, operate in plants and insects as well as in the higher animals and man. But they are very elusive and it is questionable whether they may be seen microscopically; some of them most probably not. We have concentrated on this question of visibility with what we believe to be unusually favorable material, namely, the polyhedral viruses of insects, and in particular those which produce a "wilt disease" of gypsy larvæ and "grasserie" of silkworms. It has been claimed that these diseases are caused by a chlamydozoan^{2,3} and by a *Borrellina*.⁴ Previous work by one of us revealed no organisms of etiological significance in either.^{5,6} We wish now to report briefly our comparison of diseased and normal blood made because the viruses are present in the former and absent in the latter.

Technique.

We employed many methods of fixation and staining and tried to devise new ones without finding a technique more satisfactory than

¹ Rivers, T. M., *J. Bact.*, 1927, xiv, 217.

² Wolff, M., *Mitt. Kaiser Wilhelm Inst. Landes. Bromberg*, 1910, iii, 69.

³ von Prowazek, S., *Centr. Bakt., 1. Abt., Orig.*, 1912, lxxvii, 268.

⁴ Paillot, A., *Ann. Inst. Pasteur*, 1926, xl, 314.

⁵ Glaser, R. W., *J. Agric. Research*, 1915, iv, 101.

⁶ Glaser, R. W., *Ann. Entomol. Soc. America*, 1927, xx, 319.

that of Giemsa. Various methods of vital staining were attempted but shed no light on the problem. Our optical equipment consisted of one of the latest Zeiss microscopes with lens combinations giving magnifications of from 900 to 2,400 diameters. In the examination of fresh preparations with different types of condensers a strong arc light was found indispensable. The condensers most frequently used were the paraboloid condenser which shows the reflections of colloidal particles down to 100 $m\mu$ in diameter, and the cardioid condenser (also called the cardioid ultramicroscope) which reveals the reflections of particles down to 50 $m\mu$. The ultramicroscope of Siedentopf and Zsigmondy by which one may detect reflections of particles as small as 10–15 $m\mu$ was also employed.⁷

Qualitative Comparisons.

A representative protocol follows.

Blood from early cases of gipsy moth wilt and blood from early cases of silkworm grasserie were separately collected under sterile conditions. The blood from each insect was divided into two portions. With one of these from each diseased gipsy larva, five healthy gipsy larvæ were inoculated; and with one from each diseased silkworm, five healthy silkworms were likewise injected. The healthy gipsy larvæ were collected from a lightly infested tract of woods and examined for the presence or absence of polyhedral bodies according to the well known blood method. Only those larvæ entirely free from polyhedra were used. The silkworms which we employed had been reared in the laboratory, and since the entire stock was free from grasserie, no further precaution was necessary. All the inoculated insects subsequently died of either wilt or grasserie within the expected time, showing that the blood contained the infectious agent. Controls, inoculated with blood inactivated by heat, survived. The other portion of the infectious blood from each animal was studied immediately after being drawn in both the fresh and the fixed and stained condition.

The methods of Romanowsky, of Wright, and especially of Giemsa very often brought to light, in exceedingly thin films of the diseased blood, minute single or double coccoidal bodies colored pale pink or blue. These bodies could be best seen with a 1.5 mm. apochromatic

⁷ International Critical Tables, New York, 1926, i, $\mu = \frac{1}{1,000}$ mm.; $m\mu = \frac{1}{1,000,000}$ mm.

objective, and a 10 or 20 ocular. Beside these little stained particles nothing else was visible except blood cells, unstained polyhedra, and some debris. But when normal blood was prepared and studied in an identical manner the same type of stained particles was observed. Examination with the paraboloid or cardioid condensers of fixed and stained normal and diseased blood showed many minute particles of various sizes not revealed by ordinary illumination. These were not dye particles, because Giemsa's fluid is not colloidal and the formation of precipitates was avoided. With fresh material no evidence of independent motility of the particles was ever observed when care was taken to exclude air currents from the preparations, and when vibrations were reduced to a minimum. As the diseases progressed, and the tissues commenced to disintegrate, the particles in the blood increased numerically. Up to a certain point the condition then obtaining could be imitated in normal blood by permitting it to stand for a few days until the cells disintegrated and liberated much particulate matter. Heavily diseased blood, tissues, and dead larvæ were, however, useless for finer microscopic study, owing to the rapid final lysis of all the tissues with the discharge of protein particles, fat globules, pigment granules, urates, and various other substances.

From such comparisons made between normal fresh blood and infectious fresh blood, and between normal stained blood and infectious stained blood, we have come to the conclusion that no observable qualitative distinction exists between the various particles found in the normal and in the diseased condition in either wilt disease or grasserie, and further that none of these particles resemble minute cells or organisms.

Quantitative Comparisons.

If some of the particles within the diseased insects are etiologically important, they should numerically outrank similar particles found within the normal individuals, and for this reason we attempted to secure quantitative data. That such may be of value is indicated by the fact that, in most bacterial and other infections of insects, the living causative agents are usually very numerous. This is probably due to the open form of circulation. Paillot⁸ points out that diseases

⁸ Paillot, A., *Ann. Epiphyties*, 1922, viii, 265.

in insects caused by a limited number of microorganisms, which produce injury through soluble toxins, are as yet unknown. Many counts were accordingly made of small microscopic and ultramicroscopic particles in normal blood and in blood from early cases of grasserie. We present a protocol of one experiment out of twelve.

TABLE I.

Counts of Particles in Silkworm Blood Made with the Aid of a Cardioid Condenser.

The blood from three normal and three grasserie-infected silkworms was used. Two preparations were made from each worm and the particles in ten fields of every specimen were enumerated. Lens combination: 1.5 mm. objective, and 10 ocular.

Worm	Preparation	1	2	3	4	5	6	7	8	9	10
Grasserie blood.											
A	1	165	154	83	95	115	43	195	29	42	58
	2	10	22	35	75	28	45				
B	1	24	67	68	76	55	74	22	26	52	32
	2	8	6	9	11	8	2	9	11	4	7
C	1	33	40	22	13	21	36	45	20	75	21
	2	18	26	30	43	40	35	21	9	42	48
Normal blood.											
A'	1	160	45	39	55	53	22	89	41	27	46
	2	80	51	110	60	35	75	70	19	44	38
B'	1	8	30	50	20	25	30	15	53	88	24
	2	14	7	25	38	37	2	45	40	8	56
C'	1	26	14	52	19	30	63	21	47	17	35
	2	43	7	56	50	20	23	38	29	24	25

0.1 cc. of normal silkworm blood and 0.1 cc. of blood from a silkworm infected with grasserie by inoculation 4 days previously were each diluted 100 times with sterile, distilled water. The diluted blood was then centrifuged $\frac{1}{2}$ hour at 1,000 R.P.M. to eliminate the blood cells, the larger particles, and the polyhedra. It was found in 1927,⁶ that centrifuging the virus for 4 hours at 2,000 R.P.M. did not reduce its infectivity. 1 standard drop (0.1 cc.) of the clear upper fluid from each lot was then placed on the center of each of a series of scrupulously clean cover-slips.

These were floated over mercury and dried in air, to obtain a uniform distribution of the particles present. A diaphragm was inserted into the ocular to reduce to about one-half the field of vision and the particles were counted both microscopically, and ultramicroscopically (with the aid of the cardioid condenser). This was done for ten fields on each cover-slip. In each of the twelve experiments two cover-slips representing normal blood and two representing grasserie blood were used. The microscopic shapes of the particles and their ultramicroscopic reflections seemed identical as studied in normal and diseased blood, and the size varied from about 50–100 $m\mu$ to approximately 0.5μ depending upon the type of illumination employed.

The average microscopic particle counts of diseased and normal blood were naturally considerably lower, owing to lesser visibility, than the average ultramicroscopic particle counts in the same preparations. It is significant, however, that the same ratio was found between the number of particles in the diseased and normal blood when examined in these two ways.

A statistical analysis⁹ of the determinations (Table I) shows that the average ultramicroscopic particle count for grasserie blood was 42.3 ± 3.3 , and for normal blood, 40.2 ± 2.3 . The standard deviation for grasserie blood was calculated to be 38.3 ± 2.4 , and for normal blood, 26.8 ± 1.7 . The difference in the variability was 11.4 ± 2.9 , or 3.9 times the probable error. It seems, therefore, that there is no outspoken difference in the number of ultramicroscopically visible particles existing in normal and grasserie-infected blood, although the number in the latter is slightly the more variable as one would expect in the case of animals whose tissues are undergoing such rapid degeneration.

SUMMARY.

With the techniques employed we have not been able to detect any qualitative differences between the particles visible in normal blood and in blood from cases of wilt disease and grasserie. Of the two conditions we can speak more definitely in the case of grasserie, because this we have studied quantitatively as well, that is to say, the particles visible microscopically and ultramicroscopically have been counted without bringing to light any marked difference between

⁹ Kindly made by Dr. John W. Gowen.

normal and diseased blood. This leads us to believe that the virus of wilt disease is probably invisible, and the virus of grasserie almost certainly so, when studied with the optical equipment that we have used, and that further evidence will be necessary before one can accept the chlamydozoa or the *Borrellina* as the active etiological agents. Although these two polyhedral viruses do not appear to be visibly particulate, it does not follow that other filterable viruses are not organized in this way. Each should be considered on its own merits.

EFFECT OF FORMALIN ON THE VIRUS OF VESICULAR STOMATITIS OF HORSES.

BY PETER K. OLITSKY, M.D., AND PERRIN H. LONG, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

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From time to time reports have appeared dealing with active immunization with filtrable or allied viruses that have been killed by weak solutions of formalin.

Among those recently making favorable reports are Cumming,¹ Vallée, Carré and Rinjard,² the British Foot-and-Mouth Disease Research Committee,³ and Hunt and Falk.⁴ Cumming worked with rabies virus treated less than 24 hours with 0.4 per cent formaldehyde, while Vallée and his coworkers studied foot-and-mouth disease virus exposed to 0.5 per cent formalin at 20°C. for from 4 to 7 days. The British Committee used the formalin in 0.1 per cent strength, which was added to filtered contents of foot-and-mouth disease vesicles, the mixture being kept for 48 hours at 26°C. before use. Hunt and Falk tested vaccinia material to which was added formaldehyde in 0.1 per cent concentration for 12 hours, the mixture being kept at unstated temperature. Besides these favorable reports, failures have been recorded. Thus Abramson and Gerber⁵ were unable to secure immunization with formalinized virus of poliomyelitis.

It is obvious that the subject is one of wide interest and importance, for if active immunization can be secured with filtrable viruses, altered by this simple chemical means so that they are incapable of inducing specific lesions, a great step forward will have been taken. Up to the present, active immunity has been secured only by the use of attenuated or modified, but still living viruses, capable of producing some, although at times very slight, specific changes in the tissues.

¹ Cumming, J. G., *J. Infect. Dis.*, 1914, xiv, 33.

² Vallée, H., Carré, H., and Rinjard, P., *Rec. méd. vet.*, 1925, ci, 297; *Rec. gén. méd. vet.*, 1926, xxxv, 129.

³ Second Progress Report of the Foot-and-Mouth Disease Research Committee, Ministry of Agriculture and Fisheries, London, 1927, 1-117.

⁴ Hunt, L. W., and Falk, I. S., *J. Immunol.*, 1927, xiv, 347.

⁵ Abramson, H. L., and Gerber, H., *J. Immunol.*, 1918, iii, 435.

Our study was made with a strain of the virus of vesicular stomatitis of horses, already described,^{6,7} which has been shown to be closely related to the virus of foot-and-mouth disease. It was designed to determine (a) whether protection could be obtained with formalinized virus, and (b) whether the protection could be construed as arising from the effects of a killed antigen.

Before proceeding with the description of experiments, we wish to recall some earlier conclusions derived from tests with antiseptics on the virus of foot-and-mouth disease.⁸ The remarkable resistance of the latter virus to certain chemicals was found to be due to the fact that the reagents coagulate the proteins of the medium in which the virus is ordinarily distributed. As a result of this coagulation, the positive charge of the virus, and its minute size, the active material is held within large coagula and prevented from coming into immediate contact with the chemicals. Similar conditions have been found to occur in the instance of the virus of vesicular stomatitis,⁷ in which the virus proved resistant to many chemicals which coagulate proteins. Furthermore, one of us (Long) has found that still another chemical, namely, neutral acriflavine in 1 per cent solution, acts as a coagulant so that the virus proves resistant when submitted to it for at least 24 hours. Since formalin is a substance which coagulates proteins, we studied the virucidal action by means of a series of controls, as shown in the following tests.

Viability of Formalinized Virus.

In the preparation of formalinized virus of vesicular stomatitis, we followed mainly the methods employed by the British Foot-and-Mouth Disease Research Committee.³ The Committee have studied the problem in connection with the virus of foot-and-mouth disease more extensively than any other recent workers.

⁶ Olitsky, P. K., Traum, J., and Schoening, H. W., *J. Am. Vet. Med. Assn.*, 1926, lxx, 147.

⁷ Olitsky, P. K., *J. Exp. Med.*, 1927, xlv, 969.

⁸ Olitsky, P. K., and Boëz, L., *J. Exp. Med.*, 1927, xlv, 815; and the Report of the American Commission to Study Foot-and-Mouth Disease, to be published by the U. S. Bureau of Animal Industry.

Preparation.—The virus consisted of the clear fluid derived from vesicles in the pads of from three to over ten guinea pigs injected 24 hours previously, as a rule, with guinea pig passage virus of vesicular stomatitis. The fluid was aspirated into a syringe and diluted 1 in 50 with phosphate buffer solution at a pH of 7.5 or 7.6. The diluted material was filtered through a Berkefeld V candle and the major portion of the filtrate was used for formalinization and the remainder for control tests of pathogenicity in guinea pigs. Six lots of filtered virus were prepared at different times. To four, 0.1 per cent neutral formalin was added, to one, 0.5 per cent, and to the sixth, 10 per cent. The formalinized virus was kept in an incubator at 26°C. for 48 hours or longer, and then tested by injection into the pads of guinea pigs.

The 0.5 and 10 per cent formalinized material proved too injurious for use: extensive necrosis (or "wet" gangrene) of the pad followed the use of the 10 per cent mixture, and a somewhat less degree of destruction, the 0.5 per cent mixture. Although the virus was killed in both instances after 2 days at 26°C., the factors of injury, confusion of specific and non-specific effects, and mortality led us to abandon the higher concentrations of formalin in favor of 0.1 per cent of this chemical, which is the concentration generally employed in preparing the so called "vaccines" of filtrable viruses. As will be shown later, even 0.1 per cent formalin is not wholly free from injurious effects—a mild edema being sometimes induced in guinea pigs. Similar effects in rabbits were observed by Hunt and Falk.⁴

Titration of Filtered Virus.—In order to determine the activity of the original filtered virus before utilizing mixtures of it with formalin for immunization tests, it was essential to show that the specimens of virus employed produced the characteristic lesion in guinea pig pads. Of three samples accurately titrated, one induced experimental vesicular stomatitis in a 1:1,000,000 dilution, another in 1:500,000, and a third in 1:100,000 dilution. The remaining samples were injected, as a routine procedure, in dilutions of 1:100 or 1:1,000, all being capable at these dilutions of producing typical lesions.

From now on we shall designate as "vaccine" the 0.1 per cent formalinized virus. We wish again to emphasize the fact already mentioned that this strength of formalin has in some animals an injurious action on the pad tissues, as shown by edema following injection. This injurious action is uninfluenced by either strong or weak virus; hence it must be ascribed to the chemical alone.

Living Virus in the Vaccine.

Tests were first made for evidence of living virus in the mixtures with formalin. After the freshly made mixtures had stood 48 hours at 26°C., 0.5 cc. was injected into each posterior pad of at least two guinea pigs. The results were as follows:

Vaccine 2.—Two guinea pigs, A and B, injected. After 4 days, A showed a single vesicle on one pad, yielding clear, serous fluid. B exhibited a similar condi-

tion on the 3rd day. Guinea Pig B was etherized, the affected pad removed and ground in buffer solution with sterile sand. The pad emulsion was injected into the pads of two normal animals. Both developed typical vesicular lesions on the following day.

Vaccine 5.—Two guinea pigs, A and B, injected. On the next day, A showed a single vesicle on the right pad, while B was unaffected. 14 days after the original inoculation, B was injected in both pads with active virus. The following day, typical vesicles had appeared on both pads.

Vaccine 6.—Four guinea pigs, A, B, C, and D, injected. 24 to 48 hours later, all showed either single vesicles or no visible lesions; yet on pricking the pad with a needle, clear, serous fluid exuded. This clear fluid from Guinea Pig D was inoculated into the pads of Guinea Pig E, in which typical vesicles arose within 24 hours. Guinea Pigs A, B, and C were reinjected with active virus after 14 days, and as none responded they can be regarded as immune, a finding which confirmed our opinion that the initial lesions following the injection of vaccine were those of vesicular stomatitis.

Vaccine 7.—Three guinea pigs, A, B, and C, injected. After 24 to 48 hours, no visible lesions appeared, except edema. On pricking the pads with a needle, a clear, serous fluid exuded. Guinea Pig A was etherized, the pads removed, ground in buffer solution with sand, and the emulsion inoculated into the pads of a normal animal. The latter developed typical vesicles by the next day.

From the above four tests, it may be concluded that the so called vaccines as prepared with 0.1 per cent formalin contain living virus. In contrast, we wish to record that guinea pigs in which pad inoculation was made with 0.5 per cent and 10 per cent formalinized virus developed severe necrotic lesions, from the fluid contents of which the virus of vesicular stomatitis could not be recovered. One may infer that the virus is killed by these concentrations.

The experiments described indicate that the usual concentration of formalin (0.1 per cent) employed for the production of so called vaccines of filtrable viruses does not kill the virus of vesicular stomatitis after a contact of at least 2 days at 26°C. The fact should, however, be noted that the lesions induced by the treated virus are generally slight, and that they may be confused with edema resulting from the action of the formalin alone. Simple inspection of the inoculated site is not sufficient to determine whether the effect is wholly chemical or due in part to living virus. In order to determine this, one should prick the superficial skin with hypodermic needles so as to cause any existing fluid to exude, which fluid, or that obtained from the emulsi-

fied pad, should be reinoculated into normal animals and the effects observed. Finally, recovered guinea pigs should be subjected to a later injection of active virus to determine if immunity exists,⁹ and any pads showing suspicious lesions should be examined histologically for the presence of characteristic pathological changes and of the inclusion bodies which we have already described.¹⁰ Such has been our own routine procedure.

Since, as the tests described above show, 0.1 per cent formalin fails to kill the vesicular stomatitis virus after 48 hours contact, we next studied the point at which the infectiousness of the so called vaccine ceased.

Vaccine 7 (0.1 Per Cent Formalin).—Three guinea pigs injected with this vaccine kept for 48 hours at 26°C. after its preparation, were found to harbor the virus, a fact demonstrated by the preceding protocol. Two of three animals which received the same material after it had stood for 72 hours at 26°C. also gave evidence of containing living virus, after transferring the ground pads of the two animals to normal guinea pigs, which, in turn, developed characteristic vesicles. Six guinea pigs injected with the preparation after it had stood for 96 and 120 hours at 26°C. exhibited no effects which could be attributed to living virus. These unaffected animals were reinoculated in the pads about 10 days later with active, undiluted virus. All responded promptly with characteristic vesicles.

It is plain that Vaccine 7 contained living virus for 72 hours but not for 96 hours. The fact should be noted, in addition, that a single injection into the pads of formalinized virus, unless living virus is present in it, fails to immunize the animals.

Effect of Repeated Injections of Killed Formalinized Virus.

In view of the fact that previous workers have reported the production of active immunity by means of repeated injections of formalinized vaccines in which they supposed the virus to have been killed, we studied the effect of repeated inoculations with some of our formalin-

⁹ It has already been established^{6,7} that in experimental vesicular stomatitis, the induction of vesicles, no matter how small or few in number, is followed by a solid resistance to undiluted active virus. The same conditions prevail in experimental foot-and-mouth disease of the guinea pig (Olitsky, P. K., Schoening, H. W., and Traum, J., *North American Vet.*, 1927, viii, 42).

¹⁰ Olitsky, P. K., and Long, P. H., *Proc. Soc. Exp. Biol. and Med.*, 1928, xxv, 287.

ized virus. But, as the following protocol shows, we were unable to detect any immunity in animals "vaccinated" with dead virus.

Two vaccines were selected, Nos. 2 and 3, made with 0.1 per cent formalin, which had been shown to contain living virus after 48 hours at 26°C., and which had been kept thereafter in the cold (5°C.). After 34 and 39 days respectively at this temperature, the vaccines were injected into the pads of guinea pigs. No effects of living virus could be detected by the methods already described. None of the six animals of this series proved resistant to a later injection of active virus.

The same preparations were tested again after they had been kept at 5°C. for a longer period (70 days in the case of Vaccine 2 and 75 days¹¹ in that of No. 3). Twelve guinea pigs were given a series of seven injections with one or the other vaccine, six receiving 0.5 cc. intramuscularly and six others 1 cc. intradermally and subcutaneously, that is, 0.5 cc. in each of the posterior pads. The first six injections were given at 3 day intervals, and the seventh 4 days after the sixth. 3 days after the injections had been completed, the ten surviving animals were inoculated into the pads with virus of known activity. All responded within 24 hours with characteristic vesicles.

In the following experiment it will be shown that it is possible to induce resistance to active, undiluted virus by a single intramuscular or intracutaneous injection of living, untreated virus, although under these conditions no demonstrable lesions occur.

Fluid from vesicles 24 hours old of guinea pigs inoculated with passage virus, whether diluted 1:10 or undiluted, induced no demonstrable lesions when injected intracutaneously into the shaved skin of the abdomen, or intramuscularly into the thigh. In four of the seven animals thus treated, the pads were scratched, though not inoculated, at the time of the virus injection. These pads developed no lesions.¹² All the guinea pigs resisted pad injection of undiluted, active virus from 10 to 23 days later.

The virus of vesicular stomatitis, therefore, appears to behave as do some other filtrable viruses, that is, immunity can be induced only when living virus is employed.

¹¹ The experimenters of the British Committee state that the activity of their killed foot-and-mouth disease vaccines persists in the cold for at least 197 days, and that protection is first noted 48 hours after injection.

¹² Absence of localization in the injured pad may be ascribed in these instances to the fact that the strain of virus which we used had lost its original feeble power to induce secondary lesions. The failure of the vesicular stomatitis virus to induce demonstrable lesions after intramuscular or intracutaneous injection was first noted in cattle.⁶

SUMMARY AND CONCLUSIONS.

The virus of vesicular stomatitis is not readily killed by formalin. This chemical is one of a group which coagulates the proteins of the medium in which the virus is usually contained. It has already been found⁷ that other reagents of the protein-coagulating group are not actively virucidal, and the effect of formalin in this instance is therefore characteristic of the group.

The so called formalinized vaccines which give rise to immunity can be shown to have done so because of the presence of living virus. A single injection of such so called "vaccine," or of other material containing living virus, is capable of inducing immunity in guinea pigs. No protection, however, follows a single injection of dead virus. Furthermore, repeated inoculations of virus killed by formalin likewise fail to induce resistance against subsequent injections of the living virus.

It is concluded, with respect to the virus of vesicular stomatitis, that the use of formalin has failed to solve the problem of active immunization with dead virus.

THE ANTIGENIC COMPLEX OF STREPTOCOCCUS HÆMOLYTICUS.

IV. ANAPHYLAXIS WITH TWO NON-TYPE-SPECIFIC FRACTIONS.

BY REBECCA C. LANCEFIELD, PH.D.

(From the Hospital of The Rockefeller Institute for Medical Research.)

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Anaphylactic experiments with bacterial proteins have presented more difficulties to investigators in this field than similar experiments with proteins from other sources. This has probably been due, to a certain extent, to the primary toxicity of many bacterial proteins and, in part, to the difficulty of obtaining a sufficient quantity of concentrated material.

Zinsser and Parker (1), working with a slightly alkaline saline extract of typhoid bacilli, noted its primary toxicity and the quantitative rather than the qualitative differences between passively sensitized and normal guinea pigs when relatively large amounts of antigen were injected; whereas with smaller amounts, they did not obtain acute anaphylactic death. Using the isolated uterus method of Dale (2), they were able to eliminate the error due to toxicity of the antigen, since normal uteri did not react with typhoid antigen. They showed, in this way, that the reactions obtained with bacterial antigens were essentially similar to those obtained with such antigens as horse serum. A critical review of the previous literature on bacterial anaphylaxis is given by these authors.

The experiments reported in this and in the succeeding paper were undertaken primarily to throw some light on the question of the antigenicity of the various fractions isolated from the hemolytic streptococcus. It had already been shown that certainly two and possibly three distinct reactive substances, all non-type-specific, were present in extracts of hemolytic streptococci in addition to the type-specific protein, and that these could be separated chemically and serologically (3). Certain points of interest soon arose with regard to the anaphylactic reactions caused by these fractions, and these observations are recorded. The results presented in this paper were

obtained with the group-reactive nucleoprotein (P) and with the species-specific substance (C) which is probably a carbohydrate; while the results with the type-specific protein (M) and the non-type-specific protein (Y) sometimes associated with it are given in the succeeding paper.

Methods.

The preparation of the extracts used in these experiments has been previously described (3). Briefly, the nucleoprotein (P) was the fraction of NaOH extracts precipitable in the cold with dilute acetic acid, while the probable carbohydrate (C) was obtained from the supernatant fluids after precipitation of the other reactive fractions from their respective extracts. It was freed of protein as far as the small yields permitted. The type-specific (M) protein was obtained from HCl extracts.

The preparation of antibacterial sera was described in detail in a preceding paper and the most satisfactory method of preparing anti-P sera, also described previously (3), is summarized here for convenience. Rabbits were immunized with P by intravenous injections of 1 per cent solutions. Four daily injections of 10 mg. each were made, followed by a 3-day rest, then four more injections of 20 mg. each followed by 3 days of rest as before. A similar third series of injections of 30 mg. doses was given, then a fourth series of 40 mg. doses. If the titer of the test bleeding, taken 7 days later, was unsatisfactory, further series of 40 mg. doses were made until a satisfactory titer was obtained. The animal was then bled and the serum stored in the ice box without preservative.

Guinea pigs were sensitized passively by the intraperitoneal injection of 0.5 cc. of immune serum. Occasionally larger amounts of serum were employed and are thus recorded in the tables. Since it was impossible to obtain enough guinea pigs of exactly the same weight for use in all experiments, animals of approximately the same weight were selected for each experiment. The weights, which usually ranged between 150 gm. and 200 gm., are omitted from the tables. The sensitized animals were tested by intravenous injections of the appropriate extracts at varying intervals after sensitization, as shown in the tables. The smallest amount of extract which regularly caused acute anaphylactic death was called the minimal anaphylactic dose (M. A. D.), following the terminology of Weil (4). Since the concentration of active substances in the extracts was sometimes unknown, the dose could not always be expressed in mg. and was, therefore, in these instances given as M. A. D. for guinea pigs sensitized with the homologous serum. Sometimes both methods of recording the dosage were employed. In those instances in which it was desirable to test the anaphylactic reactions of a sensitized guinea pig with more than one substance, it was found necessary to wait 24 hours between injections in order to give the animal time to recover from the non-specific reduction of reactivity due to the first shock. The necessity for this interval has also been noted by Wells and Osborne in their study of the

relationships of certain plant proteins by means of the anaphylactic reaction (5). Control animals were always injected with doses at least as large as those used for the sensitized guinea pigs, sometimes following an intraperitoneal injection of normal rabbit serum and sometimes without this preliminary injection. They never showed symptoms of shock with the doses employed and survived indefinitely, most of them being kept under observation for several weeks.

A convenient method for making intravenous injections into guinea pigs was brought to our attention by Dr. C. H. Hitchcock. The hair was shaved from the back of the leg and a superficial longitudinal vein was easily laid bare by a small incision in the skin just above the ankle. This vein was small but was satisfactory for intravenous injections with a 25 gauge Yale needle. As many as five separate injections have been made into the same vein, although usually the veins in both legs were used for repeated injections, and occasionally similarly located veins in the forelegs were employed. All intravenous injections reported here were made in this manner.

The results of anaphylactic tests of each reactive fraction injected into guinea pigs sensitized with anti-P and with antibacterial sera are considered in order. The group-reactive P antigen is discussed first since a pure anti-P serum was available for its study. Since Zinsser and Parker reported optimal sensitization after an interval of 5 to 8 days rather than earlier, the following experiment was performed to determine the optimal time for the shocking injection after passive sensitization with this serum.

Experiment 1.—A series of guinea pigs was sensitized with anti-P serum.¹ The M. A. D. of the homologous P was determined at 24-hour intervals throughout a 4-day period. The results are tabulated in Table I.

The experiment showed that the optimal time for passive sensitization with this anti-P serum was not reached until the 2nd or 3rd day, as judged by the size of the M.A.D., and that guinea pigs became less sensitive after the 3rd day.

The next experiment was devised to show the relationships among nucleoproteins from different types of hemolytic streptococci as well as from *Streptococcus viridans* and pneumococcus.

Experiment 2.—Another series of guinea pigs was sensitized with anti-P serum. The M. A. D. of nucleoprotein from strains representing three different types of

¹ In each instance the details of preparation of immune serum are given in the tables; hence they will not be discussed in detail in the text.

hemolytic streptococcus was determined on the day after sensitization by the intravenous injection of varying amounts of a 1 per cent solution. Sensitized

TABLE I.

*Anti-P Reactions.**Time Required for Passive Sensitization with an Anti-P Serum.*

Guinea pigs sensitized with anti-P serum, R500, from a rabbit immunized with P from hemolytic streptococcus, Strain S39, Type S23.

Shocked by intravenous injections of P from homologous strain, S39			
Guinea pig	Days after sensitization	Dose	Result
		mg.	
A-1	1	5.0	+++++
A-2	1	7.5	†5 min.
A-3	2	0.5	+
A-4	2	1.0	†4 min.
A-5	3	0.5	±
A-6	3	1.0	++
A-7	3	2.0	†5.5 min.
A-8	4	2.0	+
A-9	4	5.0	†3.5 min.

20 mg. of P did not shock unsensitized control guinea pigs.

In all tables the following symbols are employed:

— indicates no shock.

±? " trace of shock.

± " slight shock.

+

++ " moderate shock.

+++ " moderately severe shock.

++++ " severe shock.

+++++ " very severe shock.

† " animal died.

hom. = homologous.

het. = heterologous.

M. A. D. = minimal anaphylactic dose.

guinea pigs were also tested with P from a strain of green streptococcus and with P from pneumococcus, Type III.² Animals surviving the first injection were

² This protein was an oxidized extract kindly supplied by Dr. Julianelle.

reinjected on the following day with a dose of hemolytic streptococcus P several times the M. A. D. in order to determine whether they had become desensitized. Control animals were not shocked by 20 mg. of P.

TABLE II.

*Anti-P Reactions.**Minimal Anaphylactic Dose of Nucleoprotein, P, from Different Strains.*

Guinea pigs passively sensitized with anti-P serum, R500, from a rabbit immunized with P from hemolytic streptococcus, Strain S39, Type S23.

Guinea pig	Shocked by intravenous injections					
	First test 1 day after sensitization			Second test 2 days after sensitization		
	Antigen: P from strain of <i>S. hemolyticus</i>	Dose	Result	Antigen: P from strain of <i>S. hemolyticus</i>	Dose	Result
B-1	S39 (hom.)	mg.	++	S39 (hom.)	mg.	±
B-2	" "	5.0	†3 min.	" "	10*	±
B-3	S43 (het.)	7.5	+	" "	10	±?
B-4	" "	5.0	†4.5 min.			
B-5	S3 "	7.5	++**			
B-6	" "		†4.5 min.			
	P from strains of related species					
B-7	V92 (<i>S. viridans</i>)	10.0	+	" "	10	†4 min.
B-8	" " "	15.0	++	S3 (het.)	20	+++
B-9	" " "	20.0	+++	S39 (hom.)	10	++++
B-10	Pneumococcus, Type III	2cc.***	†4 hrs.			

* 10 M. A. D. for 48 hours. See Table I.

** Found dead next day. Small Gram-negative bacillus in heart's blood culture.

*** Concentration of P unknown. Test made 48 hours after sensitization. A control animal was unaffected by the same dose.

Table II shows the results of this experiment. Although the three hemolytic streptococcus proteins represented three distinct serological types, the M.A.D. for guinea pigs sensitized with Serum R500 was the same; while shock, but not death, was produced by considerably

larger doses of green streptococcus protein. A pneumococcus protein injected 48 hours after sensitization resulted in delayed death. Animals surviving shock from the hemolytic streptococcus proteins were completely protected against shock on the following day with 10 mg. of homologous P, although with this interval after sensitization 1 mg. was the M.A.D.; and those surviving shock from the green streptococcus protein were partly protected against the homologous P.

The nucleoproteins were also tested in guinea pigs sensitized with antibacterial sera. Since these sera contained antibodies for all the reactive substances but in different amounts depending on the method of immunization or the individual response of the rabbit, the tests with them were partly incidental to other experiments and were collected as Experiment 3.

Experiment 3.—Guinea pigs sensitized with 0.5 cc. to 1.0 cc. of antibacterial sera were tested 1 to 6 days later by intravenous injections of nucleoprotein P in doses ranging from 4 mg. to 10 mg. The M. A. D. was usually determined. The results are recorded in Table III.

The table is self-explanatory and shows that guinea pigs passively sensitized with antibacterial sera died in acute anaphylactic shock when injected intravenously with 7.5 mg. to 10 mg. of homologous or of heterologous hemolytic streptococcus nucleoprotein. Proteins from the different strains, except possibly from the homologous strain, were approximately equally effective in producing shock. Since homologous P solutions always contained some type-specific protein M, a smaller M.A.D. was to be expected. Precipitin tests, not tabulated here, also showed the presence of P antibodies in all these sera.

Active sensitization was obtained with nucleoprotein antigens in a few instances. After numerous doubtful results, unsuccessful apparently on account of dosage or timing between injections, the following satisfactory experiment in active anaphylaxis was performed.

Experiment 4.—Three guinea pigs were given intravenous injections of P from hemolytic or from green streptococci, as shown in Table IV. 22 days later each guinea pig was reinjected with 20 mg. of hemolytic streptococcus P. Table IV summarizes the experiment.

All three guinea pigs suffered typical anaphylactic shock on reinjection with hemolytic streptococcus proteins. Although the intensity

of the shock varied with different sensitizing antigens and was most marked with the most distantly related P, still the postmortem findings

TABLE III.

Anti-P Reactions.

Effect of Nucleoprotein, P, on Guinea Pigs Sensitized with Antibacterial Sera.

Guinea pig	Sensitized with serum*		Shocked by intravenous injections			
	No.	Cc.	Days after sensitization	Antigen: P from <i>S. haemolyticus</i> strain	Dose mg.	Result
C-1	R323	1.0	6	S39 (het.)	7.5	+
C-2	"	1.0	6	" "	10.0	++++
C-3	"	1.0	6	S60 (hom.)	10.0	†4 min.
C-4	R321	0.5	3	S3 (het.)	10.0	†4 "
C-5**	R261	0.5	1	" "	4.0	—
K-1	"	0.5	1	" "	10.0	†11 min.
C-6	"	1.0	6	S60 "	5.0	+++
C-7	"	1.0	6	" "	7.5	†3.5 min.
C-8	"	1.0	6	" "	7.5	†45 "
C-9	"	1.0	6	" "	10.0	†3.5 "
M-2	Q308	1.0	3	S3 "	10.0	†3.5 "
M-3	"	1.0	2	" "	10.0	†50 "
M-6	"	1.0	3	" "	10.0	++
C-10	R446	0.5	1	S43 "	5.0	+++
C-11	"	0.5	1	" "	7.5	+++
C-12	"	0.5	1	" "	10.0	†3.5 min.

* Serum R323 was against Strain S43, Type S60.

" R321 " " " " " "

" R261 " " " S39, " S23.

" Q308 " " " S23, " "

" R446 " " " S3, " S3.

** Three other guinea pigs in this group received the same dose with the same result.

were typical of anaphylactic death in all instances. This experiment offered sufficient evidence that nucleoprotein antigens from streptococci could sensitize guinea pigs actively.

These experiments confirm the conclusions previously reached as a result of precipitin tests and absorption experiments that the nucleoprotein fraction P is common to hemolytic streptococci and that it is related to similar protein fractions of green streptococci and of pneumococci.

The anaphylactic reactions of the second non-type-specific substance were next investigated. This is referred to as the C substance and is probably a complex carbohydrate chemically similar to the type-specific soluble substances of certain other bacteria. It will be recalled however, that this fraction, while it is species-specific for hemolytic

TABLE IV.
Anti-P Reactions.
Active Sensitization with Nucleoprotein Antigens.

Guinea pig	Sensitizing injection*			Shocking injection* 22 days later			
	Antigen P from strain**	Dose	Result	Weight	Antigen P from strain	Dose	Result
D-1	S43 (<i>S. hæmolyticus</i>)	20	—	135	S43 (<i>S. hæmolyticus</i>)	20	++++ (†overnight)***
D-2	S3 " "	20	±	186	" " "	20	†50 min.
D-3	V92 (" <i>viridans</i>)	20	—	173	" " "	20	†4 . "

* Intravenous.

** Strain S43 represents *Type* S60; Strain S3 represents *Type* S3.

*** Lungs distended as in acute anaphylactic death.

streptococci, shows no type specificity whatever in its précipitin reactions. Thus, with antibacterial serum from Rabbit R446 typical disc precipitates were formed in high dilutions of C preparations from all strains of hemolytic streptococcus tested. Consequently, it was important to find out whether this antigen-antibody system could cause anaphylaxis, and the following experiment was performed to determine this point.

Experiment 5.—A series of guinea pigs was sensitized with Serum R446. On the following day the animals were tested with intravenous injections of varying amounts of "purified" C from two heterologous strains of hemolytic streptococcus. The results are given in Table V.

In this experiment typical anaphylactic shock was produced with a substance which is probably a carbohydrate. Titration of C from Strain S43 showed that 0.04 mg. was the M.A.D. for guinea pigs sensitized on the preceding day with Serum R446. Similarly, the M.A.D. of C from Strain S23 was 0.03 mg. The results exactly parallel the precipitin tests recorded in Table I of the preceding paper (3). A

TABLE V.

Anti-C Reactions.

Effect of Two Relatively Pure C Substances on Guinea Pigs Sensitized with Antibacterial Serum with a High Titer of C Precipitins. Determination of Minimal Anaphylactic Dose.

Guinea pigs sensitized with serum from a rabbit immunized with Strain S3, Type S3.

Guinea pig	Shocked by intravenous injections 1 day after sensitization		
	"Purified" C from strain	Dose	Result
E-1	S43 (het.)	0.02	++++
E-2	" "	0.04	†3.5 min.
E-3	" "	0.2	†3.5 "
E-4	" "	2.0	†4 "
E-5	S23 "	0.01	±
E-6	" "	0.03	†4 min.
E-7	" "	0.1	†5 "

The three strains used in this experiment represent three distinct types of *S. hzymolyticus*:

S3 represents Type S3.

S43 " " S60.

S23 " " S23.

crude C extracted with antiformin from another strain of Type S23 also produced typical anaphylactic death in guinea pigs sensitized with this serum. Precipitin tests with this extract were negative for M and for P substances, and paralleled the anaphylaxis results in being positive for the C substance. The results with this extract are not included in the table.

Since the C substance produced undoubted anaphylactic shock, the question of its chemical nature became of even greater interest and

theoretical importance than before. As already pointed out, it was supposed that this fraction was probably a carbohydrate, and the facts supporting this supposition have been considered previously (3), as well as the fact that the chemical analyses do not exclude the possibility that the active material is combined with protein or is protein itself. Exposure of C to active trypsin, however, did not alter its activity in the precipitin test, and the effect of such digestion on the anaphylactic reaction is shown in the following experiment.

TABLE VI.

*Anti-C Reactions.**Exposure of C Substance to Trypsin: Effect on Anaphylactic Shock.*

Guinea pigs sensitized with Serum R446 from a rabbit immunized with Strain S3, Type S3.

Guinea pig	Shocked by intravenous injections 1 day after sensitization		
	Purified C from Strain S23, Type S23, treated with:—	Dose*	Result
F-1	Inactive trypsin	0.05	+++
F-2	“ “	0.075	†3 min.
F-3	Active “	0.05	+++
F-4	“ “	0.075	†3.5 min.

* These figures may be somewhat inaccurate on account of the small volumes with which the experiment was performed.

Experiment 6.—“Purified” C substance was subjected to the action of 0.5 per cent trypsin for 50 minutes. The technique of the experiment was the same as that employed in similar experiments described in previous papers (3). The trypsin was shown to be active by a simultaneous experiment in which the type-specific protein M was completely digested under the same conditions. (See Experiment 3 in the following paper.) Titrations of the C substance exposed to inactivated trypsin (the control) and of that exposed to active trypsin were made in guinea pigs sensitized with Serum R446.

Table VI shows that trypsin did not digest the C substance or change its titer in the anaphylactic reaction under conditions which completely destroyed other reactive fractions of the hemolytic streptococcus.

A few additional experiments were made to test the reactivity of C in guinea pigs sensitized with an anti-P serum and with antibacterial sera which showed only a low titer of C antibodies in the precipitin test. Experiment 7 gives some of these results.

Experiment 7.—Two guinea pigs, sensitized 2 days earlier with an anti-P serum, were given intravenous injections of C in doses which represented 3.3 and 20 M. A. D. respectively for animals sensitized with serum potent in C antibody.

TABLE VII.

*Anti-C Reactions.**Effect of C Substances on Guinea Pigs Sensitized with:**(1) Anti-P Sera with No C Precipitins.**(2) Antibacterial Sera with Low Titer of C Precipitins.*

Guinea pig	Sensitized with serum	Kind of serum*	Shocked by intravenous injections				
			Days after sensitization	Purified C extract from strain	Dose		Result
					Mg.	M.A.D.**	
G-1	R500	Anti-P	2	S23 (hom.)	0.1	3.3	—
G-2	"	"	2	" "	0.6	20.0	—***
G-3	R322	Antibacterial	1	S43 "	0.2	5.0	—***
G-4	R264	"	1	" (het.)	0.2	5.0	—
G-5	"	"	1	S23 (hom.)	0.1	3.3	—***

* R500 was a rabbit immunized with P from Strain S39, Type S23.

R322 was a rabbit immunized with Strain S43, Type S60.

R264 " " " " " S39, " S23.

** M. A. D. in terms of Serum R446. See Table V.

*** Intravenous injections of suitable homologous P or M extracts on the following day produced typical anaphylactic death in these animals.

(See Experiment 5.) Since they were not shocked, the sensitiveness of one of these animals, G-2, was tested on the following day by an intravenous injection of nucleoprotein P.

Guinea pigs sensitized with antibacterial sera which had a low titer of C precipitins were also tested with C substances but showed no symptoms of shock, whether the C was obtained from homologous or from heterologous strains. Two of these animals, G-3 and G-5, subsequently received homologous M extracts to prove that they had been really sensitized.

This experiment emphasized the fact that this non-type-specific substance was not the same as the non-type-specific nucleoprotein P since it did not shock Guinea Pigs G-1 and G-2, sensitized with a potent anti-P serum. It also showed that C would shock guinea pigs sensitized with antibacterial sera, only if the serum had a relatively high content of C precipitins. Thus, G-3, G-4, and G-5 showed no shock when given 3 to 5 M.A.D. of C, although subsequent injections of homologous type-specific M extracts proved that this group of animals had been properly sensitized. These results parallel the precipitin reactions. This experiment will be referred to in another connection to show that the C substance is different from another non-type-specific substance (designated Y) which gave cross-reactions with certain antibacterial sera (notably, with Serum R264).

No attempts to sensitize actively with C could be made on account of the small amounts of material available.

DISCUSSION.

The anaphylactic reactions of two of the cell derivatives of *Streptococcus hæmolyticus* paralleled in every instance the precipitin reactions previously reported (3). The similarity of nucleoproteins (P) from different types as well as from different strains of hemolytic streptococci was again evident; while the partial relationships of P from related species of bacteria, such as *Streptococcus viridans* and pneumococcus, were also apparent. These conclusions are based on passive anaphylactic experiments with anti-P and with antibacterial sera and on active sensitization with P which was also accomplished.

The species-specific C substance was also effective in producing anaphylactic death in passively sensitized guinea pigs, but only if the serum showed a high titer of the non-type-specific C precipitins. Amounts as small as 0.03 mg. to 0.04 mg. were sufficient to cause death in such animals. This is an instance in which a substance, probably carbohydrate in nature, causes passive anaphylactic shock, an interesting finding in view of its chemical nature and the failure to immunize with it. It seems to be a hapten capable of reacting with antibody but not capable of stimulating antibody formation. Insufficient quantities have been available to determine its capacity to produce active anaphylaxis. A similar result has been reported since the conclusion of these experiments by Tomcsik (6) who obtained from *B. lactis*

aerogenes 5 gm. of a specific substance which was largely carbohydrate, although he could not free it of 0.9 per cent nitrogen. 0.03 mg. of this product was the minimal anaphylactic dose for guinea pigs passively sensitized with antibacterial serum, a figure strikingly similar to that obtained with the probable carbohydrate of the hemolytic streptococcus. Tomcsik's material was non-antigenic in that it did not produce active anaphylaxis in guinea pigs nor antibodies in rabbits into which it was injected. His work is analogous to that reported here with the C substance; and in both instances the final proof that the material is pure carbohydrate is still lacking, although the amount of nitrogen present in one minimal anaphylactic dose is certainly small. In the case of the streptococcus C, which contained approximately 4 per cent nitrogen, the minimal anaphylactic dose, therefore, contained only about 0.000001 gm. of nitrogen. In the present anaphylactic experiments, additional evidence that C is not a protein is the failure to digest it with trypsin; and its failure to shock guinea pigs passively sensitized with anti-P serum is further evidence that it is distinct from the non-type-specific nucleoprotein, P.

SUMMARY.

The anaphylactic reactions of two non-type-specific fractions of hemolytic streptococcus extracts parallel the precipitin reactions. The nucleoprotein, P, is a true antigen, in that it stimulates antibody production in rabbits, as shown before, and produces anaphylactic shock in guinea pigs actively as well as passively. The probable carbohydrate, C, on the other hand, does not induce antibody formation in rabbits, so far as known at present, but does produce typical anaphylactic shock in guinea pigs passively sensitized with antibacterial serum provided the serum shows a high titer of C precipitins. This is an instance of a hapten, probably carbohydrate in nature, causing anaphylactic shock in passively sensitized guinea pigs.

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THE ANTIGENIC COMPLEX OF STREPTOCOCCUS HÆMOLYTICUS.

V. ANAPHYLAXIS WITH THE TYPE-SPECIFIC SUBSTANCE.

By REBECCA C. LANCEFIELD, Ph.D.

(From the Hospital of The Rockefeller Institute for Medical Research.)

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The anaphylactic reactions caused by the group-reactive nucleoprotein P, and by the species-specific substance, C, obtained from extracts of hemolytic streptococcus have been shown in the preceding paper to parallel their precipitin reactions (1). Thus equivalent amounts of nucleoproteins derived from different types of hemolytic streptococci caused acute anaphylactic death in guinea pigs passively sensitized with anti-P serum prepared with nucleoprotein from any of these types. Moreover, nucleoproteins from related species of bacteria, *Streptococcus viridans* and pneumococcus, also caused anaphylactic death if injected into similarly sensitized guinea pigs in sufficiently large doses. This partial relationship had already been observed in precipitation and absorption experiments (2). The nucleoproteins were also capable of sensitizing guinea pigs in active anaphylactic experiments. The other non-type-specific fraction, the species-specific C substance, which seemed to be non-protein and probably carbohydrate, was also effective in producing acute anaphylactic death in passively sensitized guinea pigs. Only those antibacterial sera which showed a high titer of C antibodies in the precipitin test, however, were under the conditions of the experiments capable of sensitizing guinea pigs passively to shock with the C substance. As in the precipitin reaction, so also in the anaphylactic reaction, the C substance showed no type specificity but gave cross-reactions with sera prepared against all types of hemolytic streptococci. While the chemical data were not sufficient to prove conclusively that this substance was a carbohydrate, still they indicated that this was the case. They were substantiated by the failure of trypsin or of pepsin to affect

either the precipitin titer or the minimal anaphylactic dose of C. These two non-type-specific substances extracted from hemolytic streptococci differed both chemically and in their failure to cause reciprocal precipitin or anaphylactic reactions with their respective antisera.

The present experiments are concerned with the anaphylactic reactions of the type-specific protein, M, and with certain cross-reactions observed with some of the extracts containing M which may be due to a third non-type-specific fraction, Y.

Methods.

The preparation of extracts and of antisera has been described, and the technique of the anaphylactic test is the same as that employed with the non-type-specific fractions (1).

The protein fraction, M, which gave type-specific precipitin reactions, was tested for type-specific anaphylactic reactions as shown in Experiment 1.

Experiment 1.—Guinea pigs, sensitized with antibacterial serum, were tested with intravenous injections of homologous and of heterologous HCl extracts which contained the type-specific M. Surviving guinea pigs were reinjected on the following day with homologous M. The M.A.D. for each extract was determined, and doses were recorded as approximate multiples of the M.A.D. with a definite homologous serum. Control unsensitized guinea pigs were never shocked by these extracts. Table I shows some of the type-specific reactions.

Guinea pigs sensitized with eight different antibacterial sera showed only slight reactions when tested with intravenous injections of 2 to 50 M.A.D. of heterologous M, although the controls, similarly sensitized, died in acute anaphylactic shock when given 1 or 2 M.A.D. of homologous M. The animals surviving heterologous M, either succumbed to injection of homologous M on the following day or suffered a severe but sublethal shock. The increase in amount of homologous M required to produce death following shock with heterologous M was probably due to the presence in these extracts of traces of non-type-specific substances as impurities. Such an experiment was evidence of type-specific anaphylactic shock with the M fraction. A micro Kjeldahl nitrogen determination made on the most active lot

of HCl extract from Strain S43 showed that the M.A.D. for guinea pigs passively sensitized with homologous Serum R322 was 0.2 mg. of protein.

Active sensitization with HCl extracts was attempted; but, although doses and intervals between injections were varied considerably, no positive results were obtained. In view of the difficulty of finding the right conditions for sensitization with P, however, these negative results were not considered conclusive.

Occasional sera were encountered which gave non-type-specific anaphylactic, as well as precipitin, reactions with relatively purified HCl extracts from Strain S43. Since it was obvious that the type-specific M was not completely isolated but that traces of the other reactive fractions were almost certainly present, it was possible that these results were due to reactions of the latter with their respective antibodies which are usually present in antibacterial sera. Experiment 2 was performed to test this hypothesis.

Experiment 2.—Five guinea pigs, I-1 to I-5, sensitized with Type S23 antibacterial sera received intravenous injections of HCl extract from a strain of heterologous type, as shown in Table II. All these animals died in typical anaphylactic shock. In order to determine whether this cross-reaction was due to traces of the common nucleoprotein P, Guinea Pigs I-6 to I-12 were sensitized with a highly potent anti-P serum and were given intravenous injections of the extract used in the first part of the experiment. Such slight symptoms resulted that P was eliminated as the cause of the cross-reactions in I-1 to I-5. The extract was next tested for the presence of the other known commonly reactive substance, C, by injecting it into guinea pigs sensitized with an antibacterial serum of high potency for C antibody. Since none of these animals showed more than very slight symptoms, the common C substance was also eliminated as the cause of the cross-reactions.

All surviving guinea pigs (I-6 to I-16) were tested again, usually on the following day, with suitable homologous extracts. Typical anaphylactic death resulted, except in the cases of I-6 and I-7 which suffered +++ and ++ shock respectively, thus showing that the animals had been effectively sensitized.

Table II shows the cross-reactions of occasional Type S23 antibacterial sera with certain HCl extracts from another type. The possible explanation that traces of the common substances, P and C, were responsible for these non-type-specific reactions was eliminated in each instance by testing the extract for the presence of P and of C

TABLE I.

*Anti-M Reactions.**Type-Specific Anaphylactic Shock with Antibacterial Sera and HCl Extracts.*

Guinea pig	Sensitized with serum*		Shocked by intravenous injections				
			Test No.	Days after sensitization	HCl extract from strain**	Dose M.A.D.†	Result
	No.	Cc.					
H-1	R323	1.0	1	6	S43 (hom.)	20.0	†4.5 min.
H-2	"	1.0	1 2	6 7	S39 (het.) S43 (hom.)	2.0 1.0	± †4 min.
H-3	R321	0.5	1	1	S43 (hom.)	1.0	†4.5 min.
H-4	"§	0.5	1 2 3	1 3 4	S39 (het.) " " S43 (hom.)	5.0 10.0 2.0	++ ± †4 min.
H-5	Q866	0.5	1	2	S43 (hom.)	2.0	†4 min.
H-6	"	0.5	1 2	2 3	S39 (het.) S43 (hom.)	2.5 2.0	± ++
H-7	Q864	0.5	1	2	S43 (hom.)	2.0	†3 min.
H-8	"	0.5	1 2	2 3	S39 (het.) S43 (hom.)	2.5 4.0	± ++
H-9	R322	0.5	1	2	S43 (hom.)	2.0	†4.5 min.
H-10	"	0.5	1 2	2 3	S39 (het.) S43 (hom.)	2.5 6.0	±? +++
H-11	R324	0.5	1	2	S43 (hom.)	2.0	†4 min.
H-12	"	0.5	1 2	2 3	S39 (het.) S43 (hom.)	2.5 6.0	±? †4 min.
H-13	Q613	1.0	1	1	S39 (hom.)	1.0	†7 min.
H-14	"	1.0	1 2	1 3	S43 (het.) S39 (hom.)	50.0 2.0	++ †3.5 min.
H-15	Q317	1.0	1 2	2 3	S39 (hom.) " "	1.0 2.5	++++ +++
H-16	"	1.0	1 2	2 3	S43 (het.) S39 (hom.)	2.0 2.5	± +++

by injecting it into guinea pigs sensitized with sera of known high potency for P and for C antibodies. It was shown previously in the reciprocal experiment¹ that "purified" C did not shock guinea pigs sensitized with one of the sera which gave cross-reactions in the present experiment (Serum R264 used for I-5). This is further evidence that C is not the cause of the cross-reactions in the present instance. Since this hypothesis proved, therefore, untenable, it was necessary to assume either (1) that still another non-type-specific substance was present in the antigenic complex of the hemolytic streptococcus, or else (2) that the M substance was not strictly type-specific but that it might be nearly enough related to M from a different type to cross occasionally with an antibody which is *chiefly* specific for the latter. Considerable evidence has been accumulated indicating that the second hypothesis is incorrect. A comparison of the M.A.D. of different lots of extracts in terms of animals sensitized with homologous type serum and of others sensitized with heterologous type

¹ See Paper IV of this series, Table VII, G-4 and G-5.

* Sera R321, R322, R323, and R324 were against Strain S43, *Type* S60.

" Q864 and Q866 " " " S60, " "

" Q613 " Q317 " " " S23, " S23.

** Strain S43 represents *Type* S60.

" S39 " " S23.

† M.A.D. of S43 extract in terms of Serum R321.

" " S39 " " " " " R261 or R264.

§ 6 other guinea pigs sensitized with this serum and tested with varying combinations of these 2 antigens reacted in the same way.

In all tables the following symbols are employed:

— indicates no shock.

±? " trace of shock.

± " slight shock.

÷ " mild shock.

+ ÷ " moderate shock.

+ + ÷ " moderately severe shock.

+ + + ÷ " severe shock.

+ + + + ÷ " very severe shock.

† " animal died.

hom. = homologous.

het. = heterologous.

M.A.D. = minimal anaphylactic dose.

TABLE II.

*Anti-Y Reactions.**Non-type-Specific Anaphylactic Shock with Certain Antibacterial Sera and HCl Extracts.*

Guinea pig	Sensitized with serum No.*	Shocked by intravenous injections of HCl extract from Strain S43, Type S60			
		Days after sensitization	M.A.D. in terms of serum against		Result
			homologous Type S60	heterologous Type S23	
Antibacterial sera giving cross-reactions with HCl extracts from a strain of another type					
I-1	Q308	2	2	2	†4 min.
I-2	"	4	20	1.3	†4 "
I-3	R261	6	2	2	†4.5 "
I-4	"	1	2	2	†3 "
I-5	R264	2	2	2	†6 "
Pure Anti-P sera					
I-6	R500	6	2.5	2.5	+
I-7	"	3	10	10	±?
I-8	"	1	10	10	+
I-9	"	1	10	10	+
I-10	"	1	2	2	+
I-11	R594	1	2	2	±?
I-12	"	3	10	10	-
Antibacterial serum containing C antibody					
I-13	R446	1	1.3	1	+
I-14	"	2	15	1	±?
I-15	"	1	1	1-	±
I-16	"	2	γ**	3	±

* Guinea Pigs I-1, -2, -3, -5, -6, -11, -12 were sensitized with 1 cc. of serum; all others with 0.5 cc.

R500 was an anti-P serum against P from Strain S39, Type S23.

R594 " " " " " " " S43, " S60.

All others were antibacterial sera:

R446 was against Strain S3, Type S3

Q308 " " " S23, " S23.

R261 " " " S39, " "

R264 " " " " " "

** HCl extract from Strain S39, Type S23, given to this animal.

sera shows wide variation. For example, with the extract used to test Guinea Pig I-1, the M.A.D. in terms of *Type* S23 and *Type* S60 sera was the same, while with an extract from the same strain prepared at another time (used for I-2) the M.A.D. in terms of heterologous *Type* S23 serum was fifteen times the M.A.D. in terms of homologous *Type* S60 serum. This difference in degree of heterologous activity of different lots of extract seems significant in indicating that M itself is not responsible for the cross-reaction, but rather that some non-type-specific substance, also extracted at times by HCl, is the cause of these atypical reactions.

The following experiment shows the effect of tryptic digestion on the anaphylactic reactions of this so called Y substance as well as its effect on the type-specific M.

Experiment 3.—A concentrated HCl extract from Strain S43 was digested with 0.5 per cent trypsin for 50 minutes with the technique previously described.² Controls with heated trypsin were included. Guinea Pig O-1, sensitized with serum prepared against Strain S43, served as control and died in acute anaphylactic shock on injection of 1 M.A.D. of the undigested extract (containing heated trypsin). Guinea Pig O-2, on the contrary, sensitized in the same way, was unaffected by the same dose of digested extract, although it was proved sensitive by injection on the next day of the same amount of untreated extract which resulted in typical anaphylactic death.

The same solution, known to give cross-reactions characteristic of the non-type-specific Y, was tested in Guinea Pigs O-3 and O-4, sensitized with serum prepared against a heterologous type strain. The control, O-3, died of anaphylactic shock when given 1 M.A.D. of the undigested extract, while O-4 suffered no shock from the same amount of digested extract. On the following day, however, O-4 was killed by 1 M.A.D. of untreated extract, thus proving that it was sensitive. Table III shows these results.

The results of this experiment were clear. Tryptic digestion destroyed the type-specific M contained in S43 HCl extract, as shown by the failure of the digested material to shock O-2, which had been sensitized with the homologous serum. The animal was sensitive, for it was killed by subsequent injection with untreated extract. The control animal, O-1, which received the undigested (heated trypsin) extract, died in 3 minutes. Similar results were obtained when the

² Experiment 3 described in Paper II of this series.

digested and the undigested S43 HCl extracts were tested in guinea pigs sensitized with heterologous Serum R264: the digested extract did not affect O-4 (although subsequent injection with untreated extract caused acute anaphylactic death), while the undigested control extract was still effective in shocking Guinea Pig O-3. Like all

TABLE III.

Tryptic Digestion of HCl Extracts Containing M and Y; Effect on Anaphylactic Shock.

Guinea pig	Sensitized with serum No.*	Shocked by intravenous injections of 1 to 1.5 M.A.D.					
		Test No.	Days after sensitization	HCl extract		Result	Probable chief cause of reaction
				from strain:	treated with:		
To show tryptic digestion of M							
O-1	R322	1	1	S43 (hom.)	Inactive trypsin	†3 min.	M
O-2	"	1	1	" "	Active trypsin	—	—
		2	2	" "	—	†4 min.	M
To show tryptic digestion of Y							
O-3	R264	1	1	S43 (het.)	Inactive trypsin	†3.5 min.	Y
O-4	"	1	1	" "	Active trypsin	—	—
		2	2	" "	—	†14 min.	Y

* Guinea pigs sensitized with 0.5 cc. of serum.

Serum R322 was an antibacterial serum from a rabbit immunized with Strain S43, *Type* S60; Serum R264 was an antibacterial serum from a rabbit immunized with Strain S39, *Type* S23

the other anaphylactic tests, this corroborated the previous experiments, the results of which were tested only by the precipitin reaction, and was further evidence that these two fractions were proteins. It did not, however, serve to show that this extract really contained two substances. The following absorption experiment differentiated them in such a way as to remove the doubt as to the existence of Y.

Experiment 4.—Three guinea pigs, sensitized with untreated serum from Rabbit R261, were used as controls on the activity of the extracts: J-1 died when tested with HCl extract from a heterologous strain, while J-2 and J-3 also died when tested with HCl extract from the homologous strain. Three other guinea pigs, J-4 to J-6, were sensitized with the same serum which had been absorbed by the technique previously described (2) with a strain (S24) known to be heterol-

TABLE IV.

Antibacterial Serum, R261, Rendered Type-Specific in Its Anaphylactic Reactions by Absorption with Heterologous Bacteria, Strain S24.

Guinea pig	Sensitized with Serum R261*		Shocked by intravenous injections				
	Cc.	Preliminary treatment of serum	Test No.	Days after sensitization	HCl extract from strain	Dose M.A.D. **	Result
J-1	0.5	None (control)	1	1	S43 (het.)	2	†3 min.
J-2	0.5	" " †	1	1	S39 (hom.)	1—?	†60 "
J-3	0.5	" " §	1	1	" "	1	†5 "
J-4	0.9	Absorbed	1	1	S39 (hom.)	1—?	+++
J-5	0.9	"	1	1	S43 (het.)	2	—
			2	2	S39 (hom.)	2—	†3.5 min.
J-6	0.45	"	1	1	S43 (het.)	10	—
			2	2	S39 (hom.)	2+	†5.5 hrs.

* Serum R261 was from a rabbit immunized with Strain S39, Type S23.

** All M.A.D. in terms of Serum R261.

† J-2 controls J-4 and J-5.

§ J-3 " J-6.

ogous to all the other strains used in this experiment. J-4, tested with the homologous HCl extract, suffered severe shock but recovered since too small a test dose was used; J-5 and J-6 were first tested with the same heterologous extract which had killed J-1, but they were unaffected by it. The next day, however, they succumbed to homologous HCl extract, thus showing that they had been sensitized. All necessary controls were negative. Table IV summarizes the results.

The serum used in this experiment sensitized animals to shock with heterologous HCl extracts, but after it was absorbed with entirely

heterologous bacteria, it induced only type-specific anaphylactic reactions. Guinea Pigs J-5 and J-6, sensitized with absorbed serum, withstood respectively 2 and 10 M.A.D. of heterologous HCl extract. Subsequent injection on the following day with the homologous extract resulted in acute anaphylactic death in the case of J-5 and in delayed death in the case of J-6, which, however, had been sensitized with only half as much absorbed serum as the other animals. The absorption method, therefore, eliminated the confusing cross-reactions and brought out type-specific anaphylactic as well as precipitin reactions, thus showing that M and Y must be different substances.

Tables V to VIII, inclusive, show how much was accomplished towards demonstrating type-specific anaphylactic reactions in this serum and in others which crossed with heterologous HCl extracts, by the method of desensitizing guinea pigs with extracts of various kinds and subsequently testing with the homologous extract containing M. This *in vivo* saturation of antibodies was comparable, in part, to the *in vitro* absorption described above. The results are somewhat complicated in this instance, however, by the necessity of using extracts which, although they contained one reactive substance in excess, contained in addition traces of others as impurities. Table V shows desensitization with one of these extracts.

Experiment 5.—Guinea Pigs K-1, K-2, and K-3 were sensitized with antibacterial serum, R261, the same serum used in absorption experiment, No. 4. On the following day at hourly intervals, five subcutaneous injections each of 10 mg. of NaOH extract (chiefly P) from heterologous Strain S3 were given to K-2 and K-3. 1 hour after the last injection, 20 mg. of this heterologous extract, given to K-2 intravenously, caused no symptoms, although 10 mg., given to the sensitized but otherwise untreated control K-1, caused typical anaphylactic death. At the same time, 20 mg. of the homologous NaOH extract (containing M as well as P) caused immediate death when given intravenously to the treated K-3.

This simple experiment showed that a sensitized guinea pig (K-2) was desensitized by subcutaneous injections of P so that it no longer reacted to twice the amount of P required to kill a similarly sensitized animal (K-1) which had not received these subcutaneous injections. Guinea Pig K-3, desensitized in the same way as K-2, was, however, killed by 20 mg. of homologous NaOH extract. Since this always contained the type-specific M, in addition to P, the experiment showed

that desensitization to P was accomplished by subcutaneous injections of P without desensitizing the animal to homologous M.

The effect of such desensitization with NaOH extracts on subsequent shock with HCl extracts was tested in Experiment 6.

TABLE V.

Antibacterial Serum, R261, Rendered Type-Specific in Its Anaphylactic Reactions. Effect of Desensitizing with Heterologous NaOH Extract on Subsequent Shock with Heterologous and with Homologous NaOH Extract.

Guinea pigs sensitized with 0.5 cc. of serum, R261, from a rabbit immunized with Strain S39, Type S23.

Guinea pig	Desensitization of guinea pig prior to intravenous injections	Shocked by intravenous injections 1 day after sensitization				
		NaOH extract from strain	Dose	Result	Probable chief cause of reaction	Remarks
K-1	None (control)	S3 (het.)*	mg. 10**	†11 min.	P	
K-2	5 subcutaneous injections totaling 50 mg. (5 M.A.D.) of NaOH extract from heterologous strain, S3, 1 day after sensitization	" "	20	±?†	—	Desensitized to P
K-3	Same as K-2	S39 (hom.)	20	†4 min.	M	Not desensitized to hom. M

* Strain S3 represents Type S3.

** 1 M.A.D.

† A subsequent test on this animal with homologous M (HCl extract), following a test immaterial to this experiment, resulted in severe shock.

Experiment 6.—Guinea Pigs L-1 to L-4 were sensitized with another Type S23 serum which induced cross-anaphylactic reactions with HCl extracts from a heterologous type. L-1 and L-2 were controls on the activity of the extracts: L-1 succumbed to an intravenous injection of heterologous HCl extract, and L-2 to homologous HCl extract. 2 days after sensitization at hourly intervals L-3 and L-4 were given subcutaneous and intraperitoneal injections of NaOH ex-

tracts from a heterologous strain increasing in dosage from 5 mg. to 16.5 mg. until a total of 50 mg. had been administered. The following day the heterologous HCl

TABLE VI.

Antibacterial Serum, R264, Rendered Type-Specific in Its Anaphylactic Reactions. Effect of Desensitizing with NaOH Extract, from a Strain Heterologous to the Serum and to All Test Extracts, on Subsequent Shock with Heterologous and with Homologous HCl Extracts.

Guinea pigs sensitized with 0.5 cc. of serum, R264, from a rabbit immunized with Strain S39, Type S23.

Guinea pig	Desensitization of guinea pig prior to intravenous injections	Shocked by intravenous injections							Remarks
		Test No.	Days after sensitization	HCl extract from strain*	Dose M.A.D. in terms of serum No.**		Result	Probable chief cause of reaction	
					R 264	R 321			
L-1	None (control)	1	2	S43 (het.)	2	2	†6 min.	Y	L-3 and L-4 show a certain amount of desensitization to Y but not to hom. M
L-2	“ “	1	1	S39 (hom.)	1	0	†5 “	M	
L-3	5 subcutaneous and intraperitoneal injections, totaling 50 mg. (5 M.A.D.) of NaOH extract from heterologous Strain S3, 2 days after sensitization	1	3	S43 (het.)	2	2	—	—†	
L-4	Same as L-3	1	3	“ “	4	4	+++	Y	
		2	4	S39 (hom.)	5	0	†3.5 min.	M	

* Strain S3 represents Type S3.

" S43 " " S60.

" S39 " " S23.

** Serum R264 was against Strain S39, Type S23.

" R321 " " " S43, " S60.

† Found dead next morning; blood culture sterile.

extract was titrated by giving the desensitized guinea pigs injections of 2 and 4 M.A.D. of this extract. The smaller dose produced no shock in L-3, and the larger dose produced moderately severe, but not fatal, shock in L-4. The latter

animal was killed by 5 M.A.D. of homologous HCl extract given intravenously the next day.

This experiment showed that NaOH extracts contained substances which could desensitize guinea pigs to a certain extent to shock with heterologous HCl extract containing the non-type-specific Y. It seems probable that this is due to the presence of the Y substance in both extracts. A similar experiment was performed with another *Type S23* serum, as shown in Table VII.

Experiment 7.—This experiment differed from Experiment 6 in the use of *Type S23* serum prepared against another strain. The first four animals sensitized with this serum served as controls on the activity of the extracts. M-5 was desensitized by subcutaneous and intraperitoneal injections of NaOH extract from an entirely heterologous strain by the same method as L 3 and L-4 in the last experiment. The following day it survived an injection of more than 2 M.A.D. of heterologous HCl extract but was killed on the next day by the homologous HCl extract. M-6 was desensitized by one intravenous injection of 10 mg. of heterologous NaOH extract, a usually fatal dose, which did not kill this animal. The following day it showed very slight symptoms on injection of heterologous HCl extract but died the next day after an injection of homologous HCl extract. See Table VII for details of the experiment.

In this experiment, which is similar to Experiment 6, desensitization with entirely heterologous NaOH extract given subcutaneously and intraperitoneally or intravenously, resulted in some degree of desensitization with respect to the heterologous HCl extract which previously had killed animals sensitized with this serum. These animals, however, were not desensitized to the homologous HCl extract, for both were killed by intravenous injections of homologous HCl extracts, which in the case of M-6 was in a dose comparable in size to the dose of heterologous HCl extract used on the preceding day. Here again the desensitization was probably due to Y contained in both NaOH and HCl extracts.

In the next experiment the animals were desensitized by preliminary injections of heterologous HCl extract instead of NaOH extract and the effect of desensitization tested for both these extracts as well as for the homologous HCl extract.

Experiment 8.—A series of guinea pigs was sensitized with 1 cc. of the same serum used in the last experiment. Two of these animals served as controls on

TABLE VII.

Antibacterial Serum, Q308, Rendered Type-Specific in Its Anaphylactic Reactions.

Effect of Desensitizing with NaOH Extract, from a Strain Heterologous to the Serum and to All Test Extracts, on Subsequent Shock with Heterologous and with Homologous HCl Extracts.

Guinea pigs sensitized with 1 cc. of serum, Q308, from a rabbit immunized with Strain S23, Type S23.

Guinea pig	Desensitization of guinea pig prior to intravenous injections	Shocked by intravenous injections							
		Test No.	Days after sensitization	Extract from strain*	Kind of extract	Dose M.A.D. in terms of serum No.**		Result	Probable chief cause of reaction
						Q308	R322		
M-1	None (control)	1	2	S43 (het.)	HCl	1	7.5	†4 min.	Y
M-2	" "	1	3	S3 "	NaOH	1±†	1	†3.5 "	P
M-3	" "	1	2	" "	"	1±	1	†50 "	"
M-4	" "	1	1	S39 (hom.)	HCl	1	0	†4 "	M
M-5	5 subcutaneous and intraperitoneal injections, totaling 150 mg. (15 M.A.D.), of NaOH extract from heterologous Strain S3, 1 day after sensitization	1	2	S43 (het.)	"	2.7	20	—	—
		2	3	S39 (hom.)	"	10	0	†3.5 min.	M
M-6	None (desensitized by intravenous injections)	1	3	S3 (het.)	NaOH	1±†	1	++	P
		2	4	S43 "	HCl	1.3	10	±	Y
		3	5	S39 (hom.)	"	1.5	0	†30 min.	M

* Strain S3 represents Type S3.

" S43 " " S60.

" S39 " " S23.

** Serum Q308 was against Strain S23, Type S23.

" R322 " " " S43, " S60.

† 10 mg.

the effectiveness of the antigens; N-1 was killed by an intravenous injection of 2 M.A.D. of heterologous S43 HCl extract, and M-4 by 1 M.A.D. of homologous S39 HCl extract.

Guinea Pig N-3 was desensitized 2 days after sensitization by subcutaneous injections of 15 M.A.D. (in terms of both homologous and of heterologous sera) of heterologous S43 HCl extract. The next day this animal gave practically no reaction to an intravenous injection of 4 M.A.D. of the same extract, while it

TABLE VIII.

Antibacterial Serum, Q308, Rendered Type-Specific in Its Anaphylactic Reactions. Effect of Desensitizing with Heterologous HCl Extract on Subsequent Shock with Heterologous NaOH Extract and with Heterologous and Homologous HCl Extracts.

Guinea pigs sensitized with 1 cc. of serum, Q308, from a rabbit immunized with Strain S23, Type S23.

Guinea pig	Desensitization of guinea pig prior to intravenous injections	Shocked by intravenous injections							
		Test No.	Days after sensitization	Extract from strain*	Kind of extract	Dose* M.A.D. in terms of serum No.		Result	Probable chief cause of reaction
						Q308	R322		
N-1	None (control)	1	2	S43 (het.)	HCl	2	2	†4 min.	Y
M-4	" "	1	1	S39 (hom.)	"	1	0	†4 "	M
N-3	3 subcutaneous injections, totaling 15 M.A.D. of S43 HCl extract 2 days after sensitization	1	3	S43 (het.)	"	4	4	±	Y
		2	4	S39 (hom.)	"	5	0	†4 min.	M
N-4	None (desensitized by intravenous injections)	1	2	S43 (het.)	"	0.7	5	++	Y
		2	4	" "	"	4.7	35	—	—
		3	5	S3 "	NaOH	2±	2**	—	—
		4	6	S39 (hom.)	HCl	5	0	†22 min.	M

* See foot-notes * and **, Table VII.

** 20 mg. See Guinea Pigs M-2, M-3, and M-6, Table VII, for controls on the M.A.D. of this extract.

died in 4 minutes on the following day after an intravenous injection of 5 M.A.D. of homologous S39 HCl extract.

Guinea Pig N-4 was desensitized by intravenous injections of heterologous S43 HCl extract. 2 days after sensitization, 0.7 M.A.D. of this solution caused ++ shock; 2 days later 4.7 M.A.D. of the same solution produced no effect; and the next day, 2 M.A.D. of NaOH extract from an entirely different strain, S3, caused

no symptoms. On the following day, however, the animal died a typical anaphylactic death, though delayed to 22 minutes, following an intravenous injection of 5 M.A.D. of homologous S39 HCl extract. Table VIII gives these results.

This experiment was the reciprocal of Experiments 6 and 7. In the latter, heterologous NaOH extract was used to desensitize against HCl extract from a heterologous strain of still another type. In Experiment 8 the reverse procedure was employed: the heterologous HCl extract was used to desensitize against the NaOH extract. In both instances, a certain degree of reciprocal desensitization was obtained without as much desensitization for the homologous HCl extract. These sera, with which it had previously been impossible to demonstrate type-specific anaphylactic reactions, were, therefore, rendered type-specific in that the desensitized animals reacted slightly, or not at all, with several multiples of the M.A.D. of heterologous extracts and were killed by similar, or even smaller doses, of homologous extracts.

If no other evidence were available, the cross-desensitization between NaOH extracts and HCl extracts could be explained as due to the presence in both solutions of the non-type-specific substances, C and P, but it has already been shown in Experiment 2 that the cross-reactions of the HCl extracts cannot be due to C or to P. Since the desensitization does not prevent the reaction with the homologous extracts, it seems improbable that it could be accounted for as non-specific reduction of reactivity such as is occasioned by injection of peptones or other foreign proteins. The correct explanation, therefore, seems to be the assumption of this other non-type-specific substance, Y, in extracts of the hemolytic streptococcus.

DISCUSSION.

Type-specific anaphylactic reactions were easily obtained with the protein, M, (HCl extract) when injected into guinea pigs passively sensitized with most of the antibacterial sera used in these experiments. Four sera produced by immunizing rabbits with strains of one type were, however, encountered, which caused non-type-specific anaphylactic death in passively sensitized guinea pigs when HCl extract from a strain of another type was used for the intoxicating injection. In order to obtain type-specific anaphylactic shock in guinea pigs pas-

sively sensitized with these sera, it was necessary either (1) to absorb the serum with heterologous bacteria or (2) to desensitize the passively sensitized guinea pigs with heterologous extracts. Since the HCl extract responsible for these atypical reactions did not cause reactions in guinea pigs sensitized with potent anti-P sera or with sera potent in C antibody and, conversely, since the C substance did not cause reactions with these atypical antibacterial sera, it was necessary to assume that another non-type-specific substance was present in HCl extracts and in the hemolytic streptococcus itself. The additional fact that this so called Y substance was digested by trypsin, while C was not, eliminated the possibility that C and Y were the same substance. Reciprocal desensitization experiments with heterologous NaOH extracts and with the HCl extract which gave the atypical crossing, showed some cross-desensitization for these heterologous extracts though not for homologous, a fact probably explainable as due to the presence of varying mixtures of all the non-type-specific substances in these extracts.

Active sensitization was not accomplished with M although numerous attempts were made under varying conditions, a result which agrees with another negative result, namely, the failure so far to immunize rabbits with this substance. In both instances, however, the failure must be taken with reserve, since it is possible that some change in technique might reverse the result. Active sensitization with Y (as contained in HCl extracts) was not observed.

A certain amount of additional information as to the nature and relationships of some of the reactive substances contained in the hemolytic streptococcus has been obtained by the use of the anaphylactic reaction. It is certain that the type-specific M produced typical anaphylactic shock in passively sensitized guinea pigs. *A priori*, this fact might be considered an indication that the M substance could function as an antigen in inducing antibody production: actually, all immunization experiments with rabbits and active sensitization experiments with guinea pigs yielded negative results. But in view of the additional evidence that C, which seems to be carbohydrate in nature, also shocked passively sensitized guinea pigs and never gave evidence of antibody stimulation, it seems probably that these two substances are haptens which react with antibodies produced by the

intact bacteria, when tested by means of the precipitin reaction and the passive anaphylactic reaction, but that they have no power to elicit antibodies themselves.

The facility with which acute anaphylactic death was produced by injecting bacterial extracts into passively sensitized guinea pigs was striking. Several factors are probably involved. Many investigators have found it necessary to resort to the Dale method with the excised uterus on account of the primary toxicity of the extracts to be tested. Zinsser and Parker (3) found this true in working with extracts of typhoid bacilli, and later Zinsser and Mallory (4) had the same experience with pneumococcus extracts and even with that method found that the margin between the dose which caused reactions in the normal uterus and that which caused it in the sensitive uterus was not so great as in anaphylaxis with egg albumin or with horse serum. With the hemolytic streptococcus, however, such difficulties fortunately were not encountered, and normal guinea pigs did not react to many multiples of the test doses. The fact that relatively large amounts of concentrated extracts were available and that the serological activity, as well as the number and kind of substances present, was capable of titration in most instances contributed to the success of these experiments. An analysis of the antibodies present in the immune sera used for passive sensitization was also made by means of the precipitin test, with the result that sera with known antibody content were used in the anaphylactic experiments and the control of the results was, therefore, increased.

SUMMARY.

1. Type-specific anaphylactic shock was produced with HCl extracts of *Streptococcus hæmolyticus* in guinea pigs passively sensitized with antibacterial sera.
2. With occasional sera and certain HCl extracts, type-specific shock was not produced unless the serum was first absorbed with heterologous bacteria or unless the guinea pigs were desensitized with heterologous extracts before testing with the homologous extract. The findings indicated that this was due to the presence of the non-type-specific substance which has been provisionally designated as Y.
3. Tryptic digestion destroyed the ability of HCl extracts containing

the type-specific M substance and the non-type-specific Y substance to produce anaphylactic shock in passively sensitized guinea pigs.

4. Active sensitization was not accomplished with the type-specific M. It seems probable, therefore, that this substance is a hapten, reacting with antibodies but not stimulating their production after separation from the bacterial cell.

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THE PROPERTIES OF THE BACTERICIDAL SUBSTANCE IN MILK.

By F. S. JONES, V.M.D.

(From the Department of Animal Pathology of The Rockefeller Institute for Medical Research, Princeton, N. J.)

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From the work of many it has been established that fresh raw milk will inhibit for a time the growth of a variety of organisms.

Thus Hesse (1) noted that raw milk suppressed the growth of *B. typhosus* and the cholera vibrio. Park (2) recorded a decrease in the number of organisms in milk stored at 42°F. for 24 hours, a moderate increase being found when the sample was kept at 50°F. The inhibitory property became less efficient when the milk was kept at room temperature. Rosenau and McCoy (3) likewise studied the phenomenon and concluded that there is diminution in the number of organisms during the first 8 or 10 hours of incubation with a rapid growth thereafter. The action was more prolonged but less intense at 15°C. Jones and Little (4) working with the mastitis streptococcus always recorded definite inhibition during the first 4 hours of incubation and frequently multiplication did not take place during 6 or 8 hours.

Many views have been advanced in explanation of the phenomenon. Most workers favor the opinion that the milk substance is identical with blood alexin and is directly derived from the blood. On the other hand Rosenau and McCoy suggest that the lower counts after incubation in raw milk are explicable on the ground of agglutination and he infers that phagocytosis by leucocytes contained in milk may be in part responsible for the decrease.

Stocking (5) suggests that the lack of adaptation for growth in milk of the organisms employed in the experiments may be responsible for the phenomenon. Others believe that milk contains a definite bactericidal substance. This view may be said to be supported by Heinemann (6), Chambers (7), Hanssen (8), and Jones and Little. Hanssen explains the bacterial growth-inhibitory principle on the basis of the presence of oxidizing enzymes which originate in the food and reach the udder from the circulation. Jones and Little regard the substance as one resembling alexin but originating in the udder and differing from blood alexin.

From the work of others and previous work done in this laboratory sufficient evidence exists that there is in cow's milk a substance which is capable of restraining the growth of certain bacteria for definite periods. Little is known concerning the properties of the growth-inhibitory principle. Heinemann showed that it was destroyed when boiled or heated to 60°C. for 30 minutes. Chambers noted destruction at 80° or 90°C. for 2 minutes. Hanssen records that milk heated at 63°C. for 20 minutes and 70°C. for 15 minutes still retained its inhibitory activity although 75°C. for 15 minutes inactivated the substance. Jones and Little found that 62°C. for 20 minutes failed to appreciably affect the substance although 65° or 70°C. for like periods slightly altered its effectiveness; 80°C. for the same period, or boiling for 5 minutes completely inactivated it. They also showed that whey from milk coagulated by rennet contained the inhibitory principle in practically the same concentration as the original milk.

With the aim of obtaining more information about the principle a number of observations were made.

Method.

Since the methods used in the previous work had proved satisfactory the same general procedure was adopted. The milk was drawn directly from the cleansed udder, chilled, centrifuged at high speed and thus largely freed of fat, and heated at 58°C. for 20 minutes. Heating is usually advisable to rid the milk of organisms originating in the udder. After chilling it was distributed in amounts of 1 cc. into sterile agglutination tubes containing a glass bead. The tubes were then inoculated with a standard loop of 16 hour broth culture, diluted 1:200, of the non-hemolytic mastitis streptococcus, and incubated. All tubes were shaken at half-hourly intervals during the observations. For control purposes a portion of the milk or whey was boiled for 5 minutes and distributed and inoculated in the same manner. The contents of each tube was plated after definite intervals with 10 cc. of 2 per cent agar prepared from veal infusion. The plate cultures were incubated for 24 hours at 38°C. and the colonies counted.

Time of Maximum Concentration.

It is known that the concentration of the substance in the milk of young cows may be as great as in older cows. However, it is not known how soon after parturition it reaches its maximum in the secretion. In answer to this question the following observation is cited.

Experiment 1.—The colostrum and milk from cows were tested. A sample was obtained daily and refrigerated at 2–3°C. until three were on hand. They were then tested. The milk and colostrum in this experiment were heated at 60°C. for 20 minutes before they were inoculated. The protocol in Table I represents the findings in one instance.

TABLE I.

The Effect of Colostrum and Milk during Early Lactation on the Mastitis Streptococcus.

	Streptococci present after incubation at 38°C.				
	At once	After 2 hrs.	After 4 hrs.	After 6 hrs.	After 8 hrs.
Day of parturition	1,152	1,404	11,456	Innumerable	Innumerable
" after "	960	768	1,536	15,296	92,160
2 days after parturition	960	896	7,168	69,220	Innumerable
Control, 3 samples combined and boiled	1,218	4,564	Innumerable	Innumerable	"
3 days after parturition	1,024	832	2,880	10,816	40,896
4 " " "	1,024	896	1,472	72,232	Innumerable
5 " " "	960	896	7,168	69,220	"
Control, 3 samples combined and boiled	1,218	6,592	Innumerable	Innumerable	"
6 days after parturition	1,088	1,024	2,368	9,408	34,560
7 " " "	1,216	894	1,152	3,392	25,792
8 " " "	1,152	1,216	960	2,048	28,800
Control, 3 samples combined and boiled	1,344	3,860	51,840	Innumerable	Innumerable

From the protocol given in Table I it is apparent that the inhibitory principle is present in the colostrum of the 1st day but is not quite as effective as after a few days. This is not surprising since colostrum is largely an accumulated product composed to a considerable extent of blood serum proteins. The inhibitory action of the blood derivative would be inactivated at the temperature (60°C.) to which the colostrum and milk were subjected before the tests. During the first 4 or 5 days the concentration in the secretion of the inhibitory principles

is more or less variable. After this time the results are more uniform. From the findings at later periods, not recorded in the protocol given, it is certain that the principle is present in about its maximum activity after the 6th or 7th day.

Distribution in the Quarters of the Udder.

It seems logical to assume that the amount of the inhibitory principle would be relatively uniform in the secretion from the various

TABLE II.

The Effect of Milk from Various Quarters on the Growth of the Mastitis Streptococcus.

		Streptococci present				
		At once	After 2 hrs.	After 4 hrs.	After 6 hrs.	After 8 hrs.
Cow 82	Right fore quarter	576	704	704	1,152	37,440
	Left " "	704	768	640	1,792	57,600
	Right hind "	640	512	768	576	15,552
	Left " "	640	640	5,632	Innumerable	Innumerable
" 07	Right fore "	704	704	768	1,600	72,000
	Left " "	576	704	2,112	14,400	Innumerable
	Right hind "	704	576	1,088	3,136	86,400
	Left " "	576	576	2,048	57,600	Innumerable
All samples combined and boiled		640	5,312	115,200	Innumerable	"

quarters. That this is not altogether true is evident from the next experiment.

Experiment 2.—The milk from each quarter of two cows was drawn into separate bottles. It was chilled, centrifuged to free it from fat, and heated at 60°C. for 20 minutes; then distributed and inoculated with the usual amount of dilute broth culture. Plate cultures were prepared as usual and the plates counted after suitable incubation. The results are given in Table II.

From the evidence submitted in Table II it is clear that the concentration of the inhibitory substance in the secretion from various quarters varies considerably. In the case of Cow 82 milk from the right hind quarter completely inhibited growth during the first 6

hours and considerable inhibition was noted during 8 hours. The milk from the left hind quarter inhibited during the first 2 hours but not thereafter. That from the other quarters was more efficient in this regard but not equal to that obtained from the right hind quarter. The same is true of the milk of Cow 07, the milk from the right fore quarter being more inhibitory than that from the others. It would appear that the milk from the right half of the udder contained more of the principle than that from the left. The secretion from four other cows showed similar variations. One might suppose the difference to be due to such factors as a more liberal inflow of blood serum to certain quarters or to an expenditure of the principle upon bacteria within the udder. But when the serum content of the milk specimen is measured by serum precipitin marked differences are not apparent. Furthermore the milk from a quarter invaded with streptococci may be equally as efficient in inhibiting growth under experimental conditions as that from uninvaded quarters.

Reactivation of the Principle.

If the inhibitory principle in milk is of amboceptor-complement nature, then it should be possible to inactivate it by heat and restore the activity by the addition of a little fresh milk. In order to test this point the following experiment was devised.

Experiment 3.—Milk from a single cow was obtained as usual. After freeing of fat, it was distributed in sterile tubes in amounts of 9 cc., and all tubes heated at 58°C. for 20 minutes. After chilling, two tubes were heated at 60°C. for 2½ hours, two others at 80°C. for 20 minutes, and a fifth was boiled for 5 minutes. 1 cc. of the milk heated at 58°C. for 20 minutes was added to the contents of one tube of milk which had been heated at 60°C. for 2½ hours, and a similar amount added to one of the tubes heated for 20 minutes at 80°C. The various portions were distributed in the small tubes and inoculated and tested as usual. The complete series then comprised milk heated at 55°C. for 20 minutes; two lots of milk heated for 2½ hours at 60°C., to one of which active milk was added; two lots of milk heated at 80°C., one of which was activated; and the boiled milk. The results of the tests are given in Table III.

The experiment was repeated with similar results. It is evident that 60°C. for 2½ hours does not completely inactivate the milk. The effect of adding the active milk is readily apparent but cannot be

regarded as reactivation since the substance in the active milk, combined with that still left after heating at 60°C. for 2½ hours, would be sufficient to give considerable inhibition. The results with the series which were heated at 80°C. are in agreement with this view. In my hands this temperature has been the lowest at which the principle becomes completely inactive, yet the addition of active milk restores to only a slight degree the inhibitory effect. In other experiments it has been possible to show that as little as 10 or 20 per cent of fresh milk added to boiled milk will influence the multiplication

TABLE III.
Experiment on the Reactivation of Heated Milk.

	Streptococci present				
	At once	After 2 hrs.	After 4 hrs.	After 6 hrs.	After 8 hrs.
Milk heated at 58°C. for 20 min.....	832	576	512	640	17,280
Milk heated at 60°C. for 2½ hrs.....	640	576	9,792	72,000	Innumerable
Milk heated at 60°C. for 2½ hrs. + 1/10 volume of 58°C. milk.....	704	576	4,032	21,880	"
Milk heated at 80°C. for 20 min.....	768	17,280	Innumerable	Innumerable	"
Milk heated at 80°C. for 20 min. + 1/10 volume 58°C. milk.....	704	14,400	"	"	"
Boiled milk.....	640	23,000	"	"	"

during the first 2 hours. It seems reasonable to assume then that the substance in milk is not inactivated by heat in the sense that complement is, but that heating sufficiently to impair the inhibitory action of the milk results in actual destruction.

Filtrability of the Principle.

Is it possible to pass the inhibitory principle through filters that hold back the formed elements of the milk? From previous work it is known that whey obtained by coagulation with rennet contains the

inhibitory substance. Since whey is readily filtered it was used in the following experiment.

Experiment 4.—Samples of milk from two cows, obtained as usual, were freed of fat, mixed, and heated at 58°C. for 20 minutes. To each 150 cc. of milk, 2.5 cc. of rennet solution (1 rennet tablet dissolved in 10 cc. of 0.85 per cent NaCl solution and passed through Berkefeld candle V) was added, and after suitable incubation the whey was collected and stored in the refrigerator at 3°C. overnight. Next morning 30 cc. portions were passed rapidly through Berkefeld candles V, N, and W under a pressure of 58 to 60 mm. The unfiltered and filtered portions and whey boiled for 5 minutes were then distributed and inoculated as usual. The results of the test are given in Table IV.

TABLE IV.
The Effect of Filtration on the Inhibitory Principle.

Whey	Streptococci present				
	At once	After 2 hrs.	After 4 hrs.	After 6 hrs.	After 8 hrs.
Untreated.....	1,984	1,728	1,792	1,792	51,860
Filtered through:					
Berkefeld V.....	2,176	1,792	1,856	2,048	12,672
" N.....	2,176	2,688	11,520	Innumerable	Innumerable
" W.....	2,026	5,568	115,200	"	"
Boiled.....	2,026	5,204	Innumerable	"	"

The bactericidal principle contained in whey readily passes through the pores of a Berkefeld candle V, and its activity is not appreciably impaired. A filter of N fineness withholds most of it, inhibition being noted only during the first 2 hours. The W candle takes out almost all since whey passed through this filter behaves in general like boiled whey.

The retention of the principle by filters can conceivably be due to adsorption. It is known that the removal of casein from milk during rennet coagulation fails to remove the inhibitory substance from the whey. The agent then is not readily adsorbed by the casein. The same holds true for the fat. The results of some experiments with other adsorbents are worthy of record.

Experiment 5.—Finely ground Lieselguhr, kaolin, bolus alba, and animal charcoal were washed repeatedly in distilled water and dried; then weighed in amounts

of 1 and 2.5 gm. and sterilized in 50 cc. centrifuge tubes. Milk from two cows was freed of fat and heated at 58°C. for 20 minutes. To specified amounts of the adsorbents, 10 cc. of milk was added and the tubes shaken vigorously. The tubes were refrigerated 1 hour, again shaken, and then centrifuged for 10 minutes. The supernatant fluids were distributed as usual, inoculated, and tested. Inasmuch as milk mixed with 2.5 gm. of adsorbent behaved in a manner identical with that containing only 1 gm., the results of adsorption with the larger amount only are given in Table V.

The data given in Table V are derived from two separate sets of observations. The tests in which kieselguhr and kaolin were used were done in duplicate with the milk from two cows, the results of

TABLE V.

The Effect of Adsorbents on the Bactericidal Property of Milk.

	Streptococci present				
	At once	After 2 hrs.	After 4 hrs.	After 6 hrs.	After 8 hrs.
Milk.....	704	512	576	576	576
" + kieselguhr.....	604	462	512	384	576
" + kaolin.....	704	576	512	512	512
Boiled milk.....	640	5,440	66,240	Innumerable	Innumerable
Milk.....	896	640	512	704	640
" + bolus alba.....	832	768	768	640	704
" + animal charcoal.....	832	1,024	37,440	Innumerable	Innumerable
Boiled milk.....	768	8,640	86,400	"	"

only one series being here given. The bolus alba and animal charcoal tests were made later and were likewise done in duplicate. For this reason control findings of two lots of untreated and two lots of boiled milk are given. All the adsorbents used are negatively charged. Those that failed to adsorb the substance are slightly acid (pH 6.2 to 6.5). The animal charcoal had a pH of 7.2.

It can be said that the inhibitory principle is not adsorbed by casein, fat, kieselguhr, kaolin, or bolus alba, although considerable is taken out by animal charcoal. In this connection it is of interest to point out that blood complement is removed by kieselguhr.

The Effect of Desiccation.

A further series of experiments was undertaken to determine whether the inhibitory principle would withstand desiccation and, for the purpose, in addition to the material prepared in the laboratory commercial dried milks were employed. Several methods were employed for desiccation. Whey or milk was placed in thin collodion membranes and hung in a current of warm air. Dr. Henry Simms of this Department dried certain material by means of low pressure distillation. But the most efficient method was *in vacuo* over sulfuric acid in the refrigerator. This last method usually took 6 or 7 days. The dried material went into solution readily.

TABLE VI.

The Presence of the Bactericidal Substance in Commercial Dried Milk.

	Streptococci present				
	At once	After 2 hrs.	After 4 hrs.	After 6 hrs.	After 8 hrs.
Milk powder A.....	768	8,640	72,000	Innumerable	Innumerable
" " " boiled 5 min.....	704	2,304	72,000	"	"
" " B.....	832	576	704	704	57,600
" " " boiled 5 min.....	640	2,432	46,080	Innumerable	Innumerable

Experiment 6.—13 gm. of two brands of commercial milk powder was dissolved in 87 cc. of sterile distilled water. The milk was then freed of fat by centrifugation and both products heated at 58°C. for 20 minutes, a necessary precaution since both contained organisms. They were then distributed, inoculated, and tested as usual. The results are given in Table VI.

The differences in the concentration of the principle in the two samples are sharp. Product A was as good a culture medium when heated at 58°C. as it was when boiled. It appears that the principle was completely destroyed during the drying process. Product B had a well defined bacterial inhibitory action which was destroyed by boiling.

With milk or whey dried in the laboratory the results showed that the inhibitory principle would withstand drying but they were not as

striking as with commercial product B. This is brought out in Experiment 7.

Experiment 7.—Fat-free milk heated at 58°C. for 20 minutes was distributed in thin layers in sterile wide mouth bottles and desiccated over H_2SO_4 *in vacuo*. Low pressure was maintained by the use of an oil pump twice daily. Between evacuations the jars were stored at a temperature of 3° or 4°C. Under these conditions 7 days was required for complete drying. The residue before use was dissolved in sterile distilled water and the product tested in the usual manner. Some of the material was tested as soon as possible and the remainder in the form of dry residue stored in the room and dissolved and tested 18 days later. The results are given in Table VII.

TABLE VII.
The Effect of Desiccation on the Bactericidal Principle.

	Streptococci present				
	At once	After 2 hrs.	After 4 hrs.	After 6 hrs.	After 8 hrs.
Sample tested shortly after desiccation.....	640	896	576	4,316	72,000
The same boiled for 5 min.....	768	6,912	86,400	Innumerable	Innumerable
Sample after 18 days storage in the room.....	256	320	9,472	51,840	"
The same boiled for 5 min.....	320	9,216	72,000	Innumerable	"

The milk used in the experiment would completely inhibit growth for 6 hours and markedly influence it for 8 hours, but the dried preparation was not quite as efficient in this regard. Storage of the dried material at room temperature led to further inactivation. Desiccation by evaporation through collodion membranes over $CaCl_2$, or by low pressure distillation, led to no better results. Some of the substance always remained in the residue but never to the same concentration as in the original material and its activity deteriorated on standing.

If it were possible to obtain the substance in a concentrated form, considerably more could be learned of its properties. When one or two volumes of alcohol is added to whey and the supernatant and residue completely freed of alcohol by low pressure distillation, both

products, added to boiled milk, fail to inhibit growth. One may assume then that alcohol destroys the principle.

It was hoped that the agent adsorbed on particles of charcoal could be redissolved in more concentrated form. Several experiments were made in which milk was adsorbed with charcoal and the charcoal mixed with dilute acids and alkali, dilute phosphate solutions, physiological sodium chloride solution, and distilled water, and permitted to stand for several days in the refrigerator, the supernatant solutions finally being added to boiled milk and tested. It was possible to obtain a little inhibition with the extracts of weak alkali, phosphate, and sodium chloride, but the results obtained were not encouraging enough to warrant further work.

DISCUSSION.

Substances contained in a complex fluid like milk may be attached to or so closely associated with some of the other components that their true behavior may be masked. Granting this the inhibitory principle must yet be regarded as a definite constituent of cow's milk which is secreted with the colostrum and early milk. Other observations not reported in this paper indicate that the substance is present in the milk throughout the lactation period. It is a characteristic ingredient of the milk, being present, according to our experience, in the secretion of all cows though it is true that its concentration varies in different animals and may even vary in the milk from different quarters of the same udder.

It has already been stated that several views are prevalent regarding the nature of the principle. Stocking's contention that milk affords an improper medium for the test organisms is open to question in view of the fact that boiling milk for 5 minutes renders it an admirable medium. Further the organism employed throughout the test is an udder inhabitant and so far as can be learned exists only in milk. The action of phagocytes is ruled out in milk that is heated at 58° or 60°C. for 20 minutes and further by the results of the filtration experiments. Agglutination of the streptococcus was not found on microscopic examination. Some have supposed the principle to be blood alexin, others that it is a ferment derived from food, and yet others that it is an alexin-like substance originating in the udder.

The third view would seem to be the most reasonable. It is known that blood alexin loses its antibacterial action when diluted, and it must be greatly diluted in milk in the light of the fact that serum globulin is present in milk in small quantities only. The milk substance is much more resistant to heat than blood alexin. Unlike alexin once its activity is impaired by heat it cannot be reactivated by active milk. It is not adsorbed by the same class of adsorbents. Colostrum, which contains a large proportion of blood serum, inhibits bacterial growth even when heated to 60°C., a temperature sufficient to inactivate blood alexin.

The inhibitory substance in milk may serve to protect the udder from the growth of many types of organisms. The usual period of bacterial inhibition for which it is responsible corresponds roughly to the period between feedings by the calf which would empty the udder under natural conditions.

SUMMARY.

Certain of the properties of the bacterial agent in milk have been studied. The substance is present in the colostrum and milk of the first few days of lactation as well as later. Its concentration varies in the secretion from various quarters of the same cow. Its activity is diminished by heat and cannot be restored again by the addition of active milk. The principle is present in whey and readily passes through the coarsest Berkefeld filter although a considerable portion is retained by N candles. The finest filter (W) completely retains it. It is adsorbed by animal charcoal but not by kaolin, kieselguhr, or bolus alba. It can be desiccated and its presence has been demonstrated in one brand of dried milk.

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BACTERIAL VARIATION IN CULTURES OF FRIEDLÄNDER'S BACILLUS.

By LOUIS A. JULIANELLE, PH.D.

(From the Hospital of The Rockefeller Institute for Medical Research.)

PLATES 36 AND 37.

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During the course of studies on the biological and immunological properties of Friedländer's bacillus, at least three sharply defined types were found (1) to exist among different strains of the organism. The types were designated A, B, C, and into one Group, X, were placed several heterogeneous strains. That Friedländer's bacillus, moreover, possesses at least two different cellular constituents both of which are important in the antigenicity of the organism was recorded in later communications (2, 3). One of these is now known to be polysaccharide and is chemically different for each of the fixed types (4, 5). The differential specificity of the types appears to depend on the polysaccharides, a class of substances which are analogous to the soluble specific substance of *Pneumococcus*, originally described by Dochez and Avery (6). The second substance is protein in nature and regardless of type derivation exhibits the undifferentiated characteristics of the species. To this extent, Friedländer's bacillus possesses an antigenic complex which is analogous to that of *Pneumococcus* (7) as already described in papers from this laboratory.

It has been pointed out by other investigators (8-14) as well as ourselves that there are certain conditions which induce in Friedländer's bacillus the development of variants, a phenomenon which is now considered as bacterial dissociation.¹ The typical colonies of Friedländer's bacillus are now identified as smooth (S) and the variant colonies as rough (R), the organisms in each instance differing not only morphologically, but biologically and antigenically. The most salient

¹ For an analytic and critical review of the literature on microbic dissociation, the reader is referred to Hadley's monograph in the *J. Infect. Dis.*, 1927, xl, 1.

differences are (2) that the S cells form colonies with a smooth surface; they are virulent, encapsulated, and type-specific; and they produce the soluble specific substance. On the other hand, the R cells form colonies with a rough surface; they are avirulent, capsule-free, and not type-specific, but species-specific; and they do not elaborate the soluble specific substance. Furthermore, sera prepared by immunization with S cells are type-specific and passively protect against fatal infection by bacilli of the same type, while anti-R sera are not type-specific and possess no demonstrable protective properties for infected mice.

Further study has since disclosed that there exist among the R variants of Friedländer's bacillus additional dissociates which present definite differences in morphology and antigenicity. Moreover, observations have been made upon some of the conditions conducive to variation, the possible reversibility of the variants to their antecedent forms, and the occurrence of variants in human infection. The present communication comprises a report of these studies.

That several morphological forms or intermediates exist between the extremes of the R and S varieties of the Friedländer's bacillus has been reported by Toenniessen, Baerthlein, and Hadley. The work of the two former writers antedated the use of the present nomenclature.

EXPERIMENTAL.

Without entering into a discussion as to what constitutes a Friedländer's bacillus, the S strains studied in this investigation were all members of the *Encapsulatus* group as determined by cultural and staining reactions. They were all Gram-negative, encapsulated bacilli, which were virulent for mice and showed great variation in sugar fermentation reactions. The strain "SC" (Type A) which was most studied in the present investigation, was isolated from the blood of a fatal case of pneumonia in man. It fermented without gas, dextrose, sucrose, maltose, mannitol, and lactose.

The R strains employed in the previous study were derived by consecutive transfer of S cells in broth to which was added a concentration of 10 per cent homologous immune serum. Plate cultures were made after each transplant until R colonies were grossly visible. In the earlier studies, the R colonies were not examined for structural differences, but at a later period, however, three different forms of R colonies were recognized.

The characteristic colonies of the S and R variety have been observed with each of the serological types of Friedländer's bacillus. A detailed

study, however, has been made of those derived from Type A only, and the data, unless otherwise stated, refer solely to this type.

I. Forms of Variants Encountered.

In addition to the S variety, three different forms of colonies have been recognized. For the sake of convenience and clarity of expression these three R variants may be designated as R1, R2, and R3. Further than gross appearance the R3 colony was not studied, because it was found only rarely and sporadically in mass cultures of R cells and particularly because it was extremely unstable so that the organisms were never obtained in pure culture. The R3 colony was of the "phantom" description, escaping notice when viewed by transmitted light on account of its marked transparency. By reflected light, with most of the light obscured, it was seen as a transparent colony with a smooth surface and an annular margin which appeared slightly raised and fringed.

(a) *Morphological Differences.*—The S colony, examined by transmitted light after 15–18 hours of growth, appears opaque, white, sharply circumscribed, markedly convex, homogeneous, and circular (see Fig. 1). When seeded heavily the colonies coalesce, and this mucoid coalescent growth is typically characteristic of S organisms. By reflected light the surface of the S colony is glistening and smooth and reflects sharply and accurately the image of objects within focal distance. The colony growth is mucoid and tenacious in consistency, and is elastic to the touch of a needle. The S organism is Gram-negative, and encapsulated (Figs. 2 and 3). The rods are short and thick and most commonly occur in either single or in diplo forms.

The R1 colony by transmitted light appears transparent, pale yellow or tan in color with a slightly indented border; it is flat with a distinct central papilla that becomes more prominent with age; it is not homogeneous, and it is more or less circular in shape (see Fig. 1). By reflected light, the surface appears uneven and glossy and reflects images definitely but in a distorted fashion. Due to the manner in which the light is reflected the center of the colony seems to be raised into a small cone. The colony growth is more discrete, never as tenacious as S, and is readily picked with a needle. Stained preparations exhibits a short, slender, almost coccoid bacillus, which is Gram-

negative and unencapsulated. The forms are often so small as to suggest morphologically *B. influenzae* (Figs. 4 and 5).

The R2 colony by transmitted light appears transparent, pale yellow or tan in color, with wavy margins; it is flat, not homogeneous, but appears matted simulating a tuft of cotton (see Fig. 1). The shape of the colony varies, but in general it is circular. By transmitted light, the surface markings suggest an oyster shell, *i.e.* irregular concentric rings with rough surface, and the reflection of images is always distorted. The colony growth is not confluent, mucoid, or tenacious and is picked readily. Stained preparations show long, slender, Gram-negative bacilli which, in young cultures, often occur in long wavy chains or threads. The organism is not encapsulated (Figs. 6 and 7).

The size of the S and the R colonies varies considerably, but in a general way, the R2 colonies are the largest, the S next in size, and the R1 are the smallest. Morphologically R2 bacillus is the longest while the R1 is the shortest. The S, on the other hand, shows the greatest dimension in breadth.

Figs. 1 to 7 demonstrate the relative differences of S, R1, and R2, both in colony formation and in microscopical appearance.

(b) *Biological Differences.*—Culturally, the S and R forms exhibit as striking differences as they do morphologically. The growth of the S cells in fluid media is viscous and accompanied by the production of soluble specific substance. The S form is always encapsulated and of marked virulence for mice, the intraperitoneal injection of 1/10 millionth cc. of a young culture (6–8 hours) causing death within 24–48 hours. In such instances, the peritoneal exudate is viscous, contains relatively few leucocytes which are frequently surrounded by a clear zone separating them from the bacilli, and phagocytosis of the organism has never been observed in normal animals.

The growth of R1 and R2 in fluid media is diffuse, non-viscous, and not accompanied by the elaboration of specific soluble substance. The organisms are not encapsulated and the virulence of both forms is extremely low, since doses as large as 0.5 cc. of a young culture frequently fail to cause fatal infection in mice. The peritoneal exudate following the injection of R1 or R2 is not viscous and contains numerous leucocytes which are able apparently to phagocyte the bacteria.

The fermentation reactions of the three strains were also studied.

The carbohydrates tested were dextrose, lactose, sucrose, maltose, and mannitol. As will be seen in Table I, both the S and the R strains fermented each of the sugars within the first 24 hours of growth, except that in the case of R1, the fermentation of lactose on the two occasions tested did not occur until the 6th day.

(c) *Antigenic Differences.*—It has already been shown in a previous communication (3) that S strains of Friedländer's bacillus induce in rabbits the formation of antibodies which agglutinate type-specifically, precipitate the corresponding specific soluble substance, and protect white mice against infection by strains of the same type. Anti-S sera may in addition contain species-specific antibody depending upon

TABLE I.

Biological Reactions of S, R1, and R2 Strains of Friedländer's Bacillus (Type A).*

Strain	Capsule	Specific soluble substance	Virulence	Phagocytosis	Fermentation reactions				
					Lactose	Dextrose	Sucrose	Mannitol	Maltose
S	+	+	+	—	+	+	+	+	+
R1	—	—	—	+	+ ^x	+	+	+	+
R2	—	—	—	+	+	+	+	+	+

* The S strain was isolated from the blood of a fatal case of pneumonia in man.

+ indicates presence.

— indicates absence.

x, fermentation was delayed to 6th day, whereas all the other fermentations occurred within 24 hours.

the duration and intensity of the immunization. Consequently, anti-S sera may cause agglutination of R cells. Anti-R sera, on the other hand, are lacking in antibodies associated with type specificity and protection, and contain only the common group antibody which reacts with R organisms derived from any of the serological types. The R strains employed in these reactions, however, were mass R cultures and represented one colony arising from the continued growth of an S strain in homologous anti-S serum.

The antigenic character of R1 and R2 was correlated with that of the original S strain from which they had arisen. Antisera were prepared by the intravenous injection of rabbits with heat-killed suspensions (2) of S, R1, and R2, respectively. With the resulting immune

sera, it was established that S was agglutinated in anti-S sera, but not in either R1, or R2, antisera, as is brought out in Table II. R1 and R2 were agglutinated in anti-S serum to a slight extent, the reaction appearing granular in contradistinction to the disc reaction occurring with S in anti-S sera (2).

TABLE II.

Cross-Agglutination Reactions with S, R1, and R2 Strains of Friedländer's Bacillus (Type A).

Strain	Immune serum					
	Anti-S		Anti-R1		Anti-R2	
	1:5	1:10	1:5	1:10	1:5	1:10
S	++++	++++	—	—	—	—
R1	++	+	++++	++++	++++	++++
R2	++	+	++++	++++	++++	++++

In this and following tables + + + + indicates complete agglutination with flocculent precipitate and clear supernatant; + + +, almost complete, supernatant clouded; ++, marked agglutination; +, slight agglutination; —, no agglutination.

TABLE III.

Cross-Agglutination Reactions with R1 and R2 Strains of Friedländer's Bacillus (Type A).

Immune serum	Strain	Final dilution of serum									
		5	10	20	40	80	160	320	640	1280	2560
Anti-R1	R1	++++	++++	++++	++++	++++	+++	+++	++	+	—
	R2	++++	++++	++++	++++	+++	+++	++	+	—	—
Anti-R2	R1	++++	++++	++++	++++	+++	+++	++	++	+	+
	R2	++++	++++	++++	++++	++++	++++	+++	+++	++	+

The immune serum of R1 was found to agglutinate both R1 and R2 to about the same extent, and conversely R2 antiserum caused an equally good agglutination of both strains. Agglutination was the typical R variety of granular sedimentation which breaks up readily upon agitation. The antigenic similarity evidenced by the agglutina-

tion reaction was further studied by means of agglutinin adsorption. R1 and R2 immune sera were adsorbed with both strains, and then tested for the presence or absence of agglutinins. The results of the experiments can be summarized briefly: Each strain (R1 and R2) adsorbs from the homologous antiserum agglutinins for both homologous and heterologous organisms; from the heterologous serum, however, antibody is removed only for the strain employed in the adsorption. In other words, R1 and R2 possess mutual agglutinating characters, but not complete, mutual adsorptive properties, as deter-

TABLE IV.

Agglutinin Adsorption Reaction.

Results of Agglutination with R1 and R2 Serum after Adsorption with R1 and R2 Strains.

Immune serum	Ad-sorbed with	Anti-gen	Final dilution of serum							
			20	40	80	160	320	640	1280	2560
Anti-R1	R1	R1	—	—	—	—	—	—	—	—
		R2	—	—	—	—	—	—	—	—
	R2	R1	++++	++++	++++	++++	++	++	+	—
		R2	—	—	—	—	—	—	—	—
Anti-R2	R1	R1	—	—	—	—	—	—	—	—
		R2	++++	++++	++++	++++	++++	++++	++	+
	R2	R1	—	—	—	—	—	—	—	—
		R2	—	—	—	—	—	—	—	—

mined by reciprocal adsorption. The data of these experiments are presented in Table IV.

As was to be expected from previous results (2) neither R1 nor R2 immune sera caused agglutination of the S strain from which they originated, nor did they cause precipitation of specific soluble substance, nor passively confer immunity upon mice infected with the antecedent S strain.

II. The Reversibility of R to S.

The R strains studied in this investigation have been remarkably stable during the 2 years they have been under observation. Since

the permanency of R becomes of paramount importance when viewed in terms of the problems of infection and epidemiology, experiments were planned to determine the reversibility of R to S. The older literature particularly with other species offers some evidence in favor of reversibility, but the objection has been raised that mass cultures were studied instead of pure line strains. More recently, however, it has been shown unimpeachably that single cell cultures of R may be caused to revert to S under proper cultural conditions. Thus Jordan (15) and Soule (16) showed interconvertibility of *B. paratyphosus* B, Levinthal (17), and Dawson and Avery (18), of *Pneumococcus*, Soule (19), of *B. subtilis*.

In the present study of reversibility, single cell strains were obtained by the technique of Avery and Leland (20). Since all experiments with pure line strains uniformly failed to bring about reversion, mass R cultures were studied instead, because such cultures might contain individual organisms with greater potentialities for reversion than the single R cells chosen at random. The observations were made with cultures derived from each of the three serological types. The methods adopted for reversion were (1) rapid transfer through meat infusion broth, (2) rapid transfer through dextrose broth, (3) growth in the supernatant culture fluid of the parent S strains, (4) growth in anti-R sera, (5) passage through normal white mice both before and after preliminary transfer through anti-R sera. The greater part of the experiments were carried out before our recognition of the two distinct forms of R variants and the mass cultures studied may have been mixtures of both forms. The results obtained with each method are briefly summarized below.

(1) *Rapid Transfer through Meat Infusion Broth.*—One strain each of both mass and single cell R cultures, derived from the three serologically different types, was carried through 90 transfers in meat infusion broth. Transplants were made two or three times daily and from time to time plates were streaked to examine colony formation and the cultures were tested for agglutination by the homologous anti-S serum. The reversion of R to S was not observed by this method.

(2) *Rapid Transfer through Dextrose Broth.*—It had been noted earlier in the study that a number of R strains which fermented

dextrose, grew in this medium in conglomerate clumps or masses strongly suggestive of a thread reaction. It is interesting to note in this connection that this phenomenon was never noticed in acid fermentation by S strains. Two mass cultures of R forms derived from a single colony were transplanted once daily in 1 per cent dextrose broth for 35 transfers. By the sixth subculture, clumped growth no longer occurred, although dextrose was still fermented. No evidence, however, was obtained of reversion.

(3) *Growth in the Supernatant of the Parent S Strain.*—18 hour broth cultures of S strains were centrifuged and the supernatant was withdrawn and rendered sterile by heating at 56°C. for 30 minutes. This was added in 10 per cent concentration to infusion broth alone and to infusion broth containing 10 per cent anti-R serum. In such media mass R cultures were transplanted twice daily for 90 transfers. At no time during the period of observation was reversion encountered.

(4) *Growth in Anti-R Sera.*—Both mass and single cell R strains derived from each of the serological types were carried twice daily through 10 per cent anti-R serum broth for 90 generations. The anti-R serum used in the different experiments was both homologous and heterologous and later, mass cultures of R organisms derived from Type A were carried through 40 transplants in 1 per cent and 5 per cent anti-R serum broth. In the earlier transplants growth always appeared in thread formation, that is, clumped in the bottom of the tube with a clear supernatant fluid. After 20 to 40 or more transplants this reaction disappeared and growth was uniformly diffused. Although in some instances the colony growth seemed to be somewhat less rough, nevertheless, reversion did not occur.

(5) *Passage through Normal White Mice.*—Each mass culture from the preceding experiment (*i.e.* after 90 transplants in anti-R serum broth) was passed through normal mice by intraperitoneal injection. As controls, two other R strains—one derived from Type A and the other from Type B—were passed through mice without preliminary growth in anti-R sera. Mice were injected with large amounts of young R cultures and the peritoneal washings reinjected into other normal mice. This was carried out with each strain through a series of 22 mice but in no instance did reversion occur.

(6) *Experiment with R1 and R2.*—The foregoing experiments on the reversion of R to S were carried out as stated with either mass cultures or pure line strains without regard to the particular form of the R variant studied. It seemed possible, however, that the question might now be answered more accurately and completely by a study of the two well defined variant forms R1 and R2. Cultures of each variety, therefore, were transferred twice daily in broth to which had been added in one series 10 per cent homologous immune serum, and in another series 10 per cent heterologous immune serum. The strains were grown in this way for 60 transplants and after 30 to 38 transfers the thread reaction had disappeared. Under these conditions it was possible to induce R1 to change to R2 but reversion of either variant to the S type was not observed.

In summarizing, then, the results of the study of reversion, it may be stated that none of the methods employed, succeeded in bringing R forms back to the S type.

This does not mean, however, that R forms are irreversible, but that under the conditions stated, the methods employed were not adequate to effect the change.

III. Some Incitants to Variation.

(a) *Experimental Derivation of R Forms.*—Mass R cultures may be experimentally derived by the continued subculture of S cells in broth to which has been added homologous immune serum, R organisms gradually appear as the S forms disappear. It is an old observation among earlier workers, however, that Friedländer's bacillus upon aging gives rise to variant colonies which differ strikingly in certain characters, the authors reporting on some or all of the properties of virulence, agglutination, and colony appearance. Our experience corroborates these results and included the isolation of R forms from aged colonies on plates and occasionally from broth cultures stored for several weeks, in which the change has spontaneously occurred.

(b) *Occurrence of R Forms in Discase.*—It is definitely known that R variants may be experimentally derived *in vitro* from S cells. The phenomenon of bacterial dissociation, however, would acquire greater significance if it could be demonstrated that the process actually takes place in the animal body during the course of infection. In order to

study this possibility, a survey was made of strains freshly isolated from a number of different pathological conditions and a careful search was made for the presence of R variants. In all, cultures from seventeen different sources were examined and these included seven cases of human pneumonia, one case of pneumonia in a guinea pig, two of liver abscesses in man, two of acute and fatal abscesses in guinea pigs, two of cystitis in man, one of infected antrum in man, and two cases of infected adenoid tissue. In five instances R forms were iso-

TABLE V.

The Occurrence of R Variants in Infections Associated with Friedländer's Bacillus.

Case No.	Source	Type	Presence of R
1	Sputum } pneumonia Autopsy }	A	Not found
2	Sputum—pneumonia	A	Not found
3	Sputum—pneumonia	A	Not found
4	Sputum—pneumonia	A	Not found
5	Sputum—pneumonia	A	Not found
6	Abscess—guinea pig (fatal)	A	Not found
7	Abscess—guinea pig (fatal)	A	Not found
8	Liver abscess	A	Not found
9	Adenoid tissue	A	Present
10	Adenoid tissue	A	Present
11	Autopsy—pneumonia (guinea pig)	B	Not found
12	Sputum—pneumonia	B	Not found
13	Infected antrum	C	Present
14	Sputum—pneumonia	Group X	Not found
15	Urine—cystitis	Group X	Not found
16	Urine—cystitis	Group X	Present
17	Liver abscess	Group X	Present

Except where stated, the strains were derived from human infections.

lated and in each they were present in mixtures of R and S. Since the occurrence of the two distinct variants, R1 and R2, was recognized only after this survey was completed, it is impossible to state the relative frequency of these two forms. However, of the R strains isolated, two were present with S organisms of Type A, one with those of Type C, and two others in association with S cells of Group X. Interestingly enough, the R strains were found not in acute infections but in chronic conditions. Thus R forms were present twice in cultures from adenoid

tissue, twice in cases of chronic cystitis, and once from a subacute antrum. Suggestive as the data are, no generalization, however, can be made from so few observations. The details of this study are recorded in Table V.

DISCUSSION.

The study of variation in cultures of Friedländer's bacillus reported in the present communication discloses three different forms of R variants. Two of the variants (R1 and R2) have been studied in detail, and they may be recognized grossly by colony formation or microscopically by the size and arrangement of the individual cells. Moreover, it has been possible to differentiate the dissociates further by serological reactions. Both variants (R1 and R2) are agglutinated in antisera prepared by injection of rabbits with either strain, but they lack the capacity of complete reciprocal agglutinin adsorption. The two R strains are markedly different from their antecedent S strain in colony appearance, morphology, virulence, and antigenicity.

A number of methods have been adopted to induce reversion of R to S. Whether the technique or its application was inadequate, the results were uniformly negative. This does not imply, however, that all R forms of Friedländer's bacillus are irreversible, but that in the case of the strains studied, the proper stimulus was not supplied by the methods used. In this connection the work of Dawson and Avery (21) offers an interesting comparison. They found one R strain of Type I pneumococcus irreversible by the identical methods which caused other R strains of the same and different types to change to the S form. In the present study, R2 has been converted to R1, while, on the other hand, R1 itself has remained unchanged following numerous transplants in homologous immune serum. Conversion of R2 to R1 and the less rough appearance of R1 colonies make it not unlikely that R1 is an intermediary form between S and R2.

The spontaneous development of R variants in S cultures of Friedländer's bacillus has been found to accompany the process of aging. Growth in immune sera *in vitro* also converts the S cells into R forms. That variation, however, is more than an *in vitro* or cultural degradation gains support from the fact that R forms have been found in cultures taken directly from foci of infection in the animal body caused

by Friedländer's bacillus. It is an interesting observation that in the cases studied R variants were found only in chronic infections and always in conjunction with S forms.

CONCLUSIONS.

1. Under proper conditions mass R cultures of Friedländer's bacillus may give rise to a number of variants which are dissimilar in colony appearance and morphology. Three such forms have been described. In two varieties, differences have been observed not only in colony formation and morphology, but also in cultural and antigenic characters.

2. None of the methods employed were adequate to cause reversion of any of the R variants to the S type. Growth of the R2 variant in its own antiserum, however, induced a change to the R1 form.

3. R forms of Friedländer's bacillus may be derived from S strains by aging or by growth in anti-S serum of the homologous type.

4. R strains may be isolated in culture directly from infection. In the cases where R forms were found, S cells were also present, and the pathological condition was of a chronic nature.

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EXPLANATION OF PLATES.

PLATE 36.

FIG. 1. Plate culture of Type A, Friedländer's bacillus, taken by transmitted light. The S colony and the two variant forms, R1 and R2, are labelled. Note opacity of S and transparency of both R varieties.

FIG. 2. Smear of peritoneal exudate of mouse infected with S. Stained with Gram, $\times 1000$. Note absence of leucocytes and presence of large capsules.

FIG. 3. Smear of S grown on agar. Gram stain, $\times 1000$. Capsule is greatly diminished.

PLATE 37.

FIG. 4. Smear of peritoneal exudate of mouse injected with R1. Stained with Gram, $\times 1000$. Note phagocytosis and lack of capsules.

FIG. 5. Smear of R1 grown on agar. Gram stain, $\times 1000$. Note size and arrangement as contrasted with S and lack of capsules.

FIG. 6. Smear of peritoneal exudate of mouse injected with R2. Stained with Gram, $\times 1000$. Note phagocytosis, lack of capsules, and length of rods.

FIG. 7 Smear of R2 grown on agar. Gram stain, $\times 1000$. Note size and arrangement and lack of capsule.





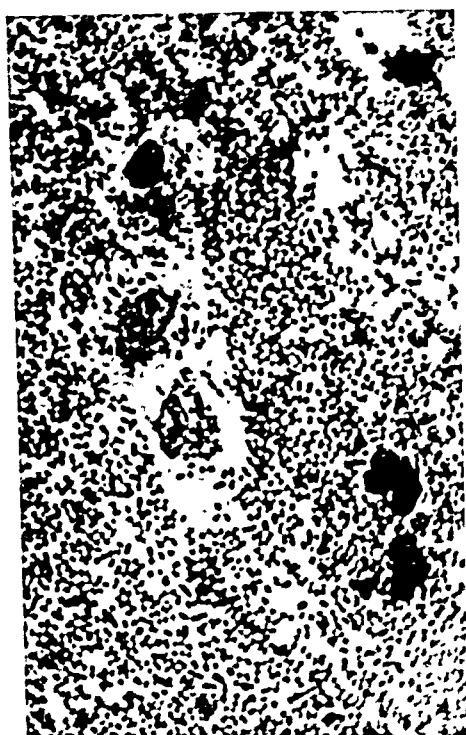


FIG. 1. (H. E. Stain.)

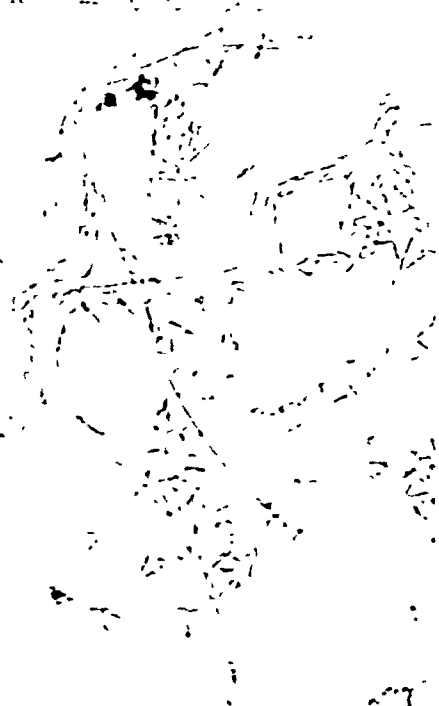


FIG. 2. (H. E. Stain.)

IS THERE AN IMMUNOLOGICAL RELATIONSHIP BETWEEN HORSE SERUM AND HORSE DANDER?

BY GEORGE F. FORSTER, PH.D.

(From the Department of Bacteriology and Immunology, Harvard University Medical School Boston, and the Department of Biology, Olivet College, Olivet, Michigan.)

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Although an antigenic relationship between horse dander and horse serum seems to have become a tacit assumption on the part of many immunologists, practically no experimental work has been aimed directly at the proof or disproof of it. The fact that a considerable proportion of asthmatics shows a cutaneous sensitiveness both to the dandruff and to the serum of a given animal species (1) has probably been responsible for this assumption, but such evidence is, of course, purely circumstantial.

Earlier Studies.

The clinical importance of horse dander as a provocative of asthma has made it the subject of much study during the last 15 or 20 years. Laboratory studies have been pursued mainly along two lines: (1) sensitization of experimental animals, usually with the object of reproducing asthmatic symptoms; (2) chemical separation of dander into its several constituent antigens.

The difficulty experienced in the sensitization of laboratory animals with horse dander has led more than one investigator to question the antigenic properties of the substance. One of these expressions of doubt bears so recent a date as 1923 (2), in spite of the considerable amount of evidence that has accumulated since horse dander was first implicated in the causation of asthma. This point has recently been reinvestigated by Longcope, O'Brien, and Perlzweig (3) and their work shows conclusively that a saline extract of horse dander is capable of sensitizing and producing anaphylactic death in guinea pigs.

Still more recently Alexander, Becke, and Holmes (4) have succeeded in sensitizing guinea pigs by exposing them to a spray of saline extract of horse dander.

Such animals showed no symptoms when again exposed after a suitable interval to this spray, but when tested by the uterine strip method in a Dale apparatus, responded with marked contraction upon the addition of dander extract to the bath.

Wodehouse (5) has made an extensive study of the separable antigens of horse dander as well as those of the hair of cats and dogs. As principal constituents of each of these he found in his weakly alkaline (N/100 KOH) extract (1) an acid-precipitable substance which he called "alkali meta-protein," (2) an "acid meta-protein," precipitated by addition of alkali to the acid filtrate from the preceding, and (3) a peptone. In addition to these he found in horse dander (4) an alcohol-soluble protein and (5) a heat-coagulable protein.

Longcope and his associates (6) have recently used more exact methods of isolation and have obtained two fractions of antigenic importance from horse dander, one of which, their protein B, makes up much the larger portion of the substances in solution. This fraction is precipitated between pH 3.2 and pH 3.8 and is probably to be identified with the "alkali meta-protein" of Wodehouse. The second fraction, their protein D, precipitates at pH 12.0, is much smaller in amount, and may possibly be the same as Wodehouse's "acid meta-protein." It is difficult to correlate these two studies exactly because the latter author did not use pH values in his description. It is further noteworthy that Longcope and his coworkers found no precipitation occurring at the isoelectric points of serum globulin or albumin.

Ratner, Jackson, and Gruehl (7) have made a very brief report which has a more direct bearing upon the results here submitted. This report states that they have obtained cross-anaphylactic reactions with horse dander and horse serum. No further details of their experimentation are given, however, except the remark that the Dale method was not relied upon as a final criterion for anaphylaxis. The writer has not encountered their further report in subsequent literature.

EXPERIMENTAL DATA.

Three routes of approach have been employed in the effort to determine whether an antigenic relationship exists between horse serum and horse dander: (1) cross-precipitation tests, rabbits being used for precipitin production; (2) cross-anaphylactic tests in guinea pigs by the uterine strip method of Dale; (3) cross-anaphylactic tests by the usual method of producing dyspnea and fatal shock in guinea pigs.

Antigens.—The dander antigens employed were prepared by various methods, but were of two principal types: (1) saline suspensions of whole dander and (2) saline extracts of dander.

In preparing the whole dander suspensions 5 gm. of the dry dander was suspended in 100 cc. of neutral 0.85 per cent NaCl by shaking with glass beads. At

first these suspensions were partially sterilized (phenolized or formalinized) to avoid severe abscess production which usually followed its intraperitoneal injection into rabbits and guinea pigs. It was found, however, that the mass of small hairs present in suspension was chiefly responsible for the abscesses. Centrifugalization removes the greater portion of hair, and abscesses were thus avoided. No difference was found in the immunizing value of the two preparations.

For the saline extract of horse dander the same proportions were used, namely, 5 gm. of dry dander per 100 cc. of neutral 0.85 per cent NaCl solution. The extract, however, had a reaction of pH 7.2 to 7.6 without buffering. A drop or two of phenol red was added to the saline to indicate the reaction. The suspension was then shaken with beads for 4 or 5 hours and centrifugalized for about an hour in order to throw down the heavier part of the material. The supernatant was then filtered either through a Berkefeld or through a Seitz filter, and was ready for use, a clear amber fluid, tinged by the phenol red. Considerable difference in antigenic value was found between the products of these two filters. This will be referred to again below.

The filtrate was found to contain as its chief antigenic constituent a protein, precipitable by dilute acetic acid, which seems to answer to the "alkali meta-protein" of Wodehouse (5) and to the protein B of Longcope, O'Brien, and Perlzweig (6). This acid-precipitable substance was obtained by adding drop by drop, stirring meanwhile, a minimal amount of ≈ 1 acetic acid for producing maximal precipitation. Two more precipitations were done for purification. That this purified fraction is fairly efficient as an antigen was demonstrated by the production of precipitin titers as high as 1-640 and by the active sensitization of one guinea pig, tested by the uterine strip method. At least one other protein was present in horse dander, but in much smaller proportions. Its isolation was not attempted.

Both whole dander suspensions and the saline extracts elicited precipitating sera of comparatively high titers. Only the extract served, however, for the precipitation tests because of the turbidity of the suspensions.

Precipitation Tests.—Ten antisera against horse dander (eight against whole dander and two against dander extract) were produced in rabbits, their titers varying from 1-160 to 1-10,240. In six of them cross-precipitation was obtained. That is to say, these six antidander sera precipitated not only homologous antigen (dander extract), but also normal horse serum. The other four antidander sera had such low titers that cross-precipitation was not attempted.

At first the reciprocal cross-reaction was not obtained, namely, the precipitation of horse dander extract by antisera against horse serum. Four of the latter antisera, ranging in titer from 1-2,560 to 1-10,640, were titrated with Berkefeld-filtered saline extract of horse dander with

negative results. Two of these antisera, when subsequently titrated against Seitz-filtered dander extract,¹ precipitated this antigen. The other two sera had not been preserved. Three additional sera have since been produced against horse serum, however, with all of which cross-precipitation has been obtained, with the Seitz-filtered antigen. Five of these antisera against horse serum have, therefore, precipitated horse dander extract.

The accompanying protocols (Tables I and II) show how the cross-precipitation tests were carried out. Forensic precipitation proportions (0.2 cc. of undiluted antiserum plus 1.0 cc. of antigen dilution, or half these quantities) were used.

Table I records the results of the titration of an antiserum against horse dander. The first group of results represents a negative control titration of undiluted antiserum against successive dilutions of an alien serum (normal human serum). Observations were made of the ring test (after 15 minutes), the flocculation test (after 1½ hours at 38° or 2 hours at room temperature), and the sedimentation test (after overnight ice box temperature). The second group of results represents a positive control titration of the antiserum against serial dilutions of homologous antigen (Berkefeld-filtered extract). The third group represents a cross-titration of the antiserum against serial dilutions of normal horse serum. The vertical column at the extreme right indicates the antiserum-saline control.

Table II shows the results of a titration of an antiserum against horse serum. The negative control antigen in this case was normal guinea pig serum, the positive control antigen normal horse serum, the antigen for cross-titration Seitz-filtered extract of horse dander.

The cross-titers obtained in the two cases represented are markedly

¹ No definite study was made to determine the reason for the difference in these two filtrates. It was observed, however, that the first portion of every Berkefeld filtrate was acid (to phenol red). The alkalinity of the dander extract neutralized the acidity of the filter after a few cc. of the filtrate had been delivered. The first few cc. came through the filter rapidly, but thereafter the filtrate was obtained very slowly. Considering the slight acidity required to precipitate the principal protein in horse dander extract, it seems reasonable to suppose that the acidity of the Berkefeld filter precipitated enough of the dander protein to clog the filter and thus increase its fineness. The Seitz filter, being neutral, offers no such problem.

TABLE I.
Titration Record of an Antiserum against Horse Dander.
 Serum 396 (against horse dander).

	1/5	1/20	1/100	1/500	1/2,000	1/10,000	1/50,000	1/200,000	Anti-serum Saline control
	turbidity + = flocculation, sedimentation, or positive ring test - = negative of two preceding								
Negative control									
Serum 396 vs. normal human serum	-	-	-	-	-	-	-	-	-
Positive control									
Serum 396 vs. horse dander extract	4+	4+	3+	2+	-	-	-	-	-
Cross-titration									
Serum 396 vs. normal horse serum	2+	3+	3+	2+	2+	+	+	+	-

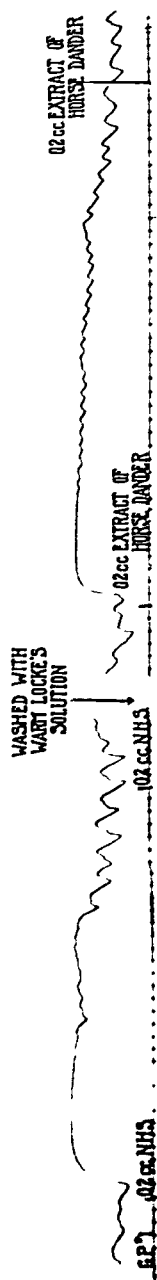


CHART 1. Guinea Pig 1. Sensitizing dose, May 18, 1.0 cc. horse dander extract (Berkefeld-filtered). Date of test, June 5. N. II. S., normal horse serum.

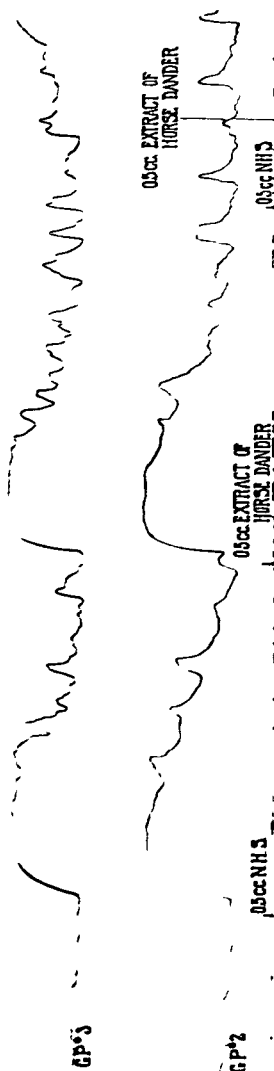


CHART 2. Guinea Pigs 2 and 3.

Guinea Pig 2. Sensitizing doses, May 17, 2.0 cc. Precipitating Serum 396 (against horse dander), intraperitoneally; May 18, 1.5 cc. same serum, intraperitoneally. Date of test, May 20. N. II. S., normal horse serum.

Guinea Pig 3. Sensitizing dose, May 18, 2.0 cc. Serum 396 intraperitoneally. Date of test, May 20.

different and this difference is characteristic. Low cross-titers were the rule in cross-precipitation of horse dander extract by antisera against horse serum.

Uterine Strip Reactions.—Both actively and passively sensitized guinea pigs were used.

Active Sensitizations.—Of the seven pigs actively sensitized to dander extract (Berkefeld-filtered) only two reacted to horse serum when tested by the uterine strip method, but these are sufficient to show that such a cross-reaction is obtainable. The kymographic record of one of these pigs (No. 1) is shown in Chart 1. This animal was sensitized by 1.0 cc. of dander extract (Berkefeld-filtered), given intraperitoneally, and was used in the test recorded 18 days later. It is noteworthy that although a reaction was obtained with horse serum, the uterus was only partially desensitized, as witnessed by the subsequent reaction to horse dander extract. This is probably explainable by the fact that horse dander contains as its chief antigenic constituent a protein which is not present in horse serum, namely the "alkali meta-protein" of Wodehouse, or protein B of Longcope *et al.* This partial desensitization of the uterus by horse serum was a regular occurrence in this type of cross-reaction (that is, including guinea pigs passively sensitized to horse dander—see below).

No pig of the eight actively sensitized to horse serum gave a response to dander extract by the Dale method.

Passive Sensitizations.—More success attended the efforts at passive sensitization. Cross-reactions by the uterine strip method of Dale were obtained in six guinea pigs passively sensitized by antidander sera from rabbits. One failure resulted, probably due to an insufficient incubation period. Two other attempts with the same antiserum that was used in this negative case were successful. Chart 2 shows the response of two of these animals (Nos. 2 and 3) to horse serum.

Passive sensitization with sera from rabbits immunized against horse serum was not attempted.

Cross-Anaphylactic Tests in Living Animals.—The results obtained in these tests are in substantial agreement with those already described.

Reactions in Guinea Pigs Sensitized to Horse Dander.—Sensitization was accomplished by a dosage of 1.0 to 2.0 cc. of centrifugalized dander suspension or of Seitz-filtered dander extract, intraperitoneally or

intracardially administered. Shock doses of 1.5 to 2.0 cc. normal horse serum were given intracardially 12 to 14 days later. Of twelve pigs thus treated ten experienced fatal shock with typical dyspnea, autopsy showing characteristic marked distention of the lungs. One

TABLE III.

Cross-Anaphylatic Tests in Guinea Pigs Sensitized to Horse Dander.

Guinea pig No.	Sensitizing injection	Shock dose	Symptoms
4	1.0 cc. centrifugalized horse dander suspension, I. P.	2.0 cc. normal horse serum, I. C.	Nose scratching, dyspnea. Death* within 3 min.
5	<i>Ibid.</i>	1.5 cc. <i>Ibid.</i>	Urination, dyspnea. Death* within 3½ min.
6	<i>Ibid.</i>	<i>Ibid.</i>	Slight dyspnea lasting a few min. Survival
7	<i>Ibid.</i>	<i>Ibid.</i>	Dyspnea. Death* within 4 min.
8	<i>Ibid.</i>	<i>Ibid.</i>	Doubtful symptoms—slight dyspnea. Survival
9	<i>Ibid.</i>	<i>Ibid.</i>	Dyspnea. Death* within 5 min.
10	<i>Ibid.</i>	<i>Ibid.</i>	Dyspnea. Death* within 3 min.
11	<i>Ibid.</i>	<i>Ibid.</i>	Dyspnea. Death* within 3½ min.
12	<i>Ibid.</i>	<i>Ibid.</i>	Dyspnea. Death* within 4 min.
13	2.0 cc. Seitz-filtered dander extract, I. C.	2.0 cc. <i>Ibid.</i>	Dyspnea. Death* within 6 min.
14	<i>Ibid.</i>	<i>Ibid.</i>	Dyspnea. Death* within 3½ min.
15	<i>Ibid.</i>	<i>Ibid.</i>	Dyspnea. Death* within 3 min.
16	No treatment	1.5 cc. <i>Ibid.</i>	Control. No symptoms
17	<i>Ibid.</i>	<i>Ibid.</i>	Control. No symptoms

* Autopsy showed typical marked distention of lungs except where contrary is indicated.

I. P. means intraperitoneal injection; I. C., intracardial.

of the two other pigs showed slight respiratory symptoms, but recovered, while the remaining one showed no definite symptoms. Two controls, receiving only the intracardial dose of horse serum showed no symptoms whatever. These results are given in more detail in Table III.

Reactions in Guinea Pigs Sensitized to Horse Serum.—Fifteen guinea

TABLE IV.
Cross-Anaphylactic Tests in Guinea Pigs Sensitized to Normal Horse Serum.

Guinea pig No.	Cross-anaphylactic tests			Dose	Symptoms
	Sensitizing injection	Shock dose	Symptoms		
18	1.0 cc. normal horse serum, I. P.	2.0 cc. horse dander extract, I. C.	Nose scratching, slight dyspnea. Survival		
19	<i>Ibid.</i>	<i>Ibid.</i>	Tremors, slight dyspnea. Survival		
20	<i>Ibid.</i>	<i>Ibid.</i>	Slight dyspnea. Survival		
21	<i>Ibid.</i>	<i>Ibid.</i> †	No symptoms		
22	<i>Ibid.</i>	<i>Ibid.</i>	Slight dyspnea. Survival		
23	<i>Ibid.</i>	<i>Ibid.</i>	Sneezing, nose scratching, dyspnea lasting several min. Survival		
24	<i>Ibid.</i>	<i>Ibid.</i>	Slight dyspnea, nose scratching. Survival		
25	<i>Ibid.</i>	<i>Ibid.</i>	No symptoms		
26	<i>Ibid.</i>	<i>Ibid.</i>	No symptoms	2.0 cc., normal horse serum, I. C.	Dyspnea. Death* within 3½ min.
27	<i>Ibid.</i>	<i>Ibid.</i>	Nose scratching, delayed dyspnea (12 min.). Death in 15 min. Lungs not distended at autopsy		
28	<i>Ibid.</i>	4.0 cc. <i>Ibid.</i>	Prostration, respiratory difficulty. Survival	4.0 cc. <i>Ibid.</i>	Typical dyspnea, lasting ½ hr. Survival
29	<i>Ibid.</i>	2.0 cc. <i>Ibid.</i>	Severe dyspnea for 11 min. Survival	2.0 cc. <i>Ibid.</i>	Moderate respiratory symptoms. Death after 13 min. Lungs not distended. Early pregnancy

Tests to determine whether desensitization had been accomplished. (On day following shock dose.)

No.	Treat.	10 cc. <i>Ibid.</i>	No symptoms	4.0 cc. <i>Ibid.</i>	Typical dyspnea. Death* in 4½ min.
30	<i>Ibid.</i>		No symptoms	4.0 cc. <i>Ibid.</i>	Death* in 4½ min.
31	<i>Ibid.</i>	20 cc. <i>Ibid.</i>	Nose scratching, prostration. Survival	20 cc. <i>Ibid.</i>	Death* in 4½ min.
32	<i>Ibid.</i>	<i>Ibid.</i>	Weakness, collapse, no respiratory symptoms, recovery. Death overnight. No autopsy	-	
33	No treatment	<i>Ibid.</i>	Control. No symptoms		
34	No treatment	<i>Ibid.</i>	Control. Death in 11 min. Lungs not distended†		
35	No treatment	<i>Ibid.</i>	Control. No symptoms		
36	No treatment	<i>Ibid.</i>	Control. No symptoms		

* Autopsy showed typical marked distention of lungs except where contrary is indicated.

† Some doubt about intracardial nature of injection.

‡ Death thought to be due to cardiac hemorrhage.

I.P. means intraperitoneal injection; I.C., intracardial.

pigs received sensitizing doses of 1.0 cc. normal horse serum intraperitoneally (see Table IV). Shock doses of 2.0 to 4.0 cc. of Seitz-filtered horse dander extract were administered 12 to 15 days later. In none of these did typical, fatal anaphylaxis occur. Three (Nos. 28, 29, 31) showed characteristic dyspnea and other symptoms and six (Nos. 18, 19, 20, 22, 23, 24) reacted more doubtfully. Six (Nos. 21, 25, 26, 27, 30, 32) failed to show any reaction which could be called anaphylactic. Four control animals were used, these receiving no sensitizing dose, but an intracardial injection of dander extract. One of these (No. 34) died a few minutes after injection, but with none of the characteristic symptoms. Autopsy showed the lungs collapsed and the pericardial cavity tightly distended with blood. It is believed that cardiac hemorrhage was responsible for death.

Five of the guinea pigs sensitized to horse serum were further used to learn whether desensitization had been brought about by injection of the horse dander extract. 24 hours after the latter injection, 2.0 to 4.0 cc. of horse serum was given intracardially to each of these (Nos. 26, 28, 29, 30, 31). For results see Table IV. Three of them (Nos. 26, 30, 31) died in typical anaphylaxis following this treatment. It is obvious that no complete desensitization resulted from any of the "shock doses" (dander extract). Partial desensitization seems to have occurred in some of the five. Thus Nos. 28 and 29 afforded the most characteristic picture of non-fatal anaphylaxis when horse dander extract was given as a "shock dose." No. 28 survived the subsequent injection of horse serum 24 hours later. No. 29 succumbed to the latter treatment, but the death was not anaphylactic, since the lungs were collapsed. Nos. 26 and 30 showed no symptoms following the "shock dose" of horse dander extract, while horse serum on the following day produced dyspnea and death with lungs distended.

DISCUSSION.

It seems evident from the foregoing results that there is an antigenic element common to horse dander and horse serum. This common antigenic substance yet remains to be isolated biochemically. That it is present in relatively small proportion in horse dander is suggested (1) by the low titer obtained in cross-precipitation tests involving an antiserum against horse serum and extract of horse dander, as well as

(2) by the failure of horse dander extract to produce fatal shock or even to desensitize completely guinea pigs which had been sensitized to horse serum. The results here obtained shed no light on the relative concentration of the common antigen in horse serum, since only a minute amount of it would probably be necessary to sensitize a guinea pig and no great amount of it would be necessary for precipitin production in a rabbit.

The work of Longcope, O'Brien, and Perlzweig (6) indicates that horse dander contains no protein precipitable at the isoelectric points of either serum globulin or serum albumin. This seems to be at variance with the results obtained in the present study since dander, if it has any antigenic element in common with serum, must contain either globulin or albumin.

It is possible that serum may be present in dander as a contaminant, as a result of exudation from cuts or sores, or from slight bleeding during the rather strenuous process of currying. If serum proteins were derived from such a source, different lots of dander would, of course, contain different proportions of them. In this study three lots of dander have been used, harmonious results being obtained from all of them. Two lots came from the horses of the Boston Fire Department and one lot from the horses of Parke, Davis and Company.

Whatever the common antigen may prove to be chemically, however, the fact of its existence seems to be attested by the results detailed herewith. Furthermore, the fact of sensitivity both to horse serum and to horse dander extract, such as is shown in a considerable percentage of horse-asthmatics is logically explained thereby.

SUMMARY.

Evidence has been submitted of the existence of a common antigenic substance in horse dander and horse serum. This evidence has been derived from three lines of study:

(a) Cross-precipitation tests involving (1) the titration of antisera against horse serum with saline extract of horse dander and (2) titration of antisera against horse dander with normal horse serum.

(b) Cross-anaphylactic tests by the uterine strip method of Dale.

(c) Cross-anaphylactic tests in living guinea pigs by the usual shock method.

It seems likely from the work here described that the common antigen is present in small proportion in horse dander. Its concentration in horse serum is not indicated by the results obtained.

The writer desires to express his appreciation to Dr. Hans Zinsser for his interest and helpful criticisms during the course of the work.

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STUDIES ON IMMUNOLOGICAL RELATIONSHIPS AMONG THE PNEUMOCOCCI.

I. A VIRULENT STRAIN OF PNEUMOCOCCUS WHICH IS IMMUNOLOGICALLY RELATED TO, BUT NOT IDENTICAL WITH TYPICAL STRAINS OF TYPE III PNEUMOCOCCI.

By JOHN Y. SUGG, EMIDIO L. GASPARI,* WILLIAM L. FLEMING, AND
JAMES M. NEILL, Ph.D.

*(From the Department of Bacteriology and Immunology, Vanderbilt University
Medical School, Nashville.)*

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INTRODUCTION.

The present paper deals with a strain of *Pneumococcus* which is related to, but not identical with typical strains of the Type III group. The general interest in strains of bacteria that possess a specific immunological relationship to one of the "fixed" types (1) of any important pathogenic group acquires special interest in the case of pneumococci in view of the fact that the specificity of *Pneumococcus* Types I, II and III furnishes the most clear-cut example in all bacteriology of the dependence of exquisite biological specificity upon the chemical constitution of the bacteria themselves (2).

In order to avoid any confusion that might arise from either the term "subgroup" or the term "atypical," the terms "Thomas strain" and "anti-Thomas serum" are used throughout the present paper to designate the "non-typical" strain and its antiserum.

It is important to point out at the beginning, that the Thomas strain is virulent, killing mice within 36 hours in doses of 1×10^{-8} cc. of plain broth culture. As shown by Tillett (3), the type-specific agglutination of the "S" forms of Type III pneumococci involves an anti-S antibody in contrast to the anti-P antibody

* Mr. Gaspari's cooperation in this work was made possible by a grant from The Henry Strong Denison Medical Foundation.

which agglutinates the "R" or degraded forms of all types of pneumococci. The characteristics which distinguish the Thomas strain from the typical Type III pneumococcus are recognized by S-anti-S reactions, and have nothing to do with those which distinguish the "S" forms (S-producing) of pneumococci from the avirulent, degraded or "R" forms (non-S-producing). Although not described in detail, the controls included in all experiments were sufficient to eliminate any complications arising from failure to differentiate the anti-S reactions from anti-P reactions. The high degree of virulence of the strain, the characteristic anti-S character of the agglutination of the bacteria and of the precipitation of young broth culture filtrates by the homologous antiserum, as well as actual tests of the non-type-specific protein-precipitating (anti-P) capacity of the antiserum, are in themselves convincing evidence that the Thomas strain is not a "degraded" form of typical Type III pneumococcus.

The Thomas strain exhibits no immunological relationship to Type I or II pneumococci with the exception of the species-specific P-anti-P relationship which is common to all pneumococci: the Thomas pneumococci are not agglutinated by Type I or II antiserum nor do these antisera confer passive protection; anti-Thomas immune serum is likewise non-reactive against Types I and II pneumococci; similarly, absorption of Type I or II antiserum with Thomas bacteria and absorption of anti-Thomas serum with Type I or II bacteria have no effect upon the type-specific antibodies.

EXPERIMENTAL

Methods.

In this investigation, five different methods have been used in testing the immune sera for the presence of the antibody specifically reactive with Type III pneumococci. These methods can be arranged in the following order of delicacy: (1) specific, passive protection of mice against virulent Type III bacteria; (2) agglutination of concentrated suspensions of heated Type III bacterial cells in salt solution; (3) agglutination of heated broth culture; (4) agglutination of unheated broth culture; (5) precipitation of solutions of the Type III specific S substance. The different procedures simply represent different methods of detecting the same anti-S antibody, and the positive results obtained when an immune serum is tested by one method, and the negative results obtained when the same serum is tested by another method, are due to differences in delicacy of the method of test.

The Relationship of the Thomas Strain to Typical Type III Strain of Pneumococci as Evidenced in Tests with Potent Anti-Type III Serum and Potent Anti-Thomas Serum.

The comparison in Table I of the immunological properties of the Thomas pneumococci with those of typical Type III pneumococci, is

TABLE I.

The Relationship of the Thomas Strain to Typical Type III Pneumococci as Evidenced in Tests with Potent Anti-Type III Horse Serum and Potent Anti-Thomas Rabbit Serum.

Dilution of serum	1. Agglutination of broth culture				2. Precipitation of S substance contained in filtrate of young broth culture				3. Type specific passive protection				
	Typical Type III strain		Thomas strain		Typical Type III strain		Thomas strain		Dose of culture cc.	Typical Type III strain		Thomas strain	
	Type III serum	Anti-Thomas serum	Type III serum	Anti-Thomas serum	Type III serum	Anti-Thomas serum	Type III serum	Anti-Thomas serum					
Undiluted	+++	+++	+++	+++	+++	+++	+++	+++	0.01	D	D	D	S
1/5	+++	++	+++	++	+++	+	++	++	0.001	D-S	D-S	D-S	S
1/20	++	0	0	++	+++	0	0	±	0.0001	S	S	S	S
1/10	±	0	0	++	+++								
1/320	0	0	0	++	+++								
1/640	0	0	0	±	+++								

0 = indistinguishable from controls; ± = slight granulation; + = granular sediment with definite particles when thoroughly shaken; ++ = bacteria sedimented in disc form but supernatant not entirely clear and disc easily broken when shaken; +++ = compact disc with supernatant entirely cleared.

D = mice died within 24 to 72 hours in all tests; D-S = protection irregular, mice surviving in some experiments and dying in others; S = mice survived in all tests. Virulence controls (without serum or with heterologous serum) dying within 72 hours when injected with 1×10^{-8} cc. of culture.

based upon tests made with the most potent anti-Thomas and the most potent anti-Type III serum which we have obtained. While there are wide variations in the individual potencies of the anti-Thomas and anti-Type III serum obtained from different animals, it is desirable to base the first comparison upon the immunological properties exhibited in immune sera containing the complete expression of the antigenic properties of the two related but different kinds of pneumococci.

As shown in Table I, the Thomas strain of pneumococci reacts with anti-Type III serum, although its agglutination, filtrate precipitation and passive protection are not as pronounced as are the corresponding reactions of typical Type III pneumococci in anti-Type III serum. Thus, in respect to the reactions in potent samples of Type III immune serum the Thomas strain appears to be related to typical Type III pneumococci in about the same way as Avery's (1) Subgroup *IIa* strains are related to typical Type II pneumococci.

However, the comparison of the two strains in the anti-Thomas serum gives a more clear-cut differentiation between the Thomas and the typical Type III strains. In the first place, the anti-Thomas serum reacts not only with the Thomas pneumococci but also with typical strains of Type III pneumococci. In the second place, the potency of the anti-Thomas serum against the Thomas bacteria is much greater than the potency of anti-Type III serum against Type III pneumococci themselves; the invariable protection of mice against 0.01 cc. of broth culture (1 million lethal doses) and the definite agglutination of broth cultures by 1/320 dilution of serum being of a much higher order of potency than that obtained with typical Type III pneumococci in anti-Type III serum whether from rabbits or hyperimmunized horses. This high degree of homologous potency of the antiserum produced by immunization with the atypical strain was a frequent occurrence in Avery's (1) work with the different Subgroup Type II pneumococci, but the reactivity of typical Type III strains in the anti-Thomas serum is in direct contrast to the lack of any reactivity of typical Type II strains in any of the different Subgroup Type II antisera.

Variations in the Relative Potencies of Different Type III Immune Sera in Respect to Antibodies Reactive with a Typical Type III Strain and Antibodies Reactive with the Thomas Strain.

The comparison in Table I of the Thomas strain with a typical Type III strain was based upon their reactions in immune sera chosen for their potency against both the Thomas strain and typical Type III strains. During the investigation, many tests have been made with a number of different Type III immune sera. The results of these tests showed a wide range of differences in the relation between the anti-Type III and the anti-Thomas potency in different anti-Type III sera. The reactivity of the serum against typical Type III pneumococci had no regular relation to its reactivity against the Thomas pneumococci. For example, one of the anti-Type III sera that was most reactive against typical Type III pneumococci did not agglutinate the Thomas strain at all after 2 hours incubation at 37°C. and only occasionally agglutinated it after storage in the ice box overnight. Similarly, one of the anti-Type III sera that was weakly reactive against typical Type III strains was as reactive against the Thomas strain as any of the sera that we tested. This lack of any relation between the anti-Thomas potency and the anti-Type III potency of the individual sera is important if it represents a difference in the relative proportion of two slightly different antibodies in the serum obtained from different horses after immunization with Type III pneumococci.

Variations in the Relative Potencies of Anti-Thomas Serum from Different Rabbits in Respect to Antibodies Reactive with Typical Type III Strains and Antibodies Reactive with the Thomas Strain.

During the investigation, ten different rabbits were immunized with heat-killed suspensions of the Thomas pneumococci. A summary of the results of the tests of the potencies of these anti-Thomas rabbit sera is presented in Table II.

An analysis of Table II reveals two important facts: (1) the anti-Thomas sera obtained from ten rabbits showed no great difference in their respective potencies against the homologous strain; (2) in spite

of the uniformly good anti-Thomas immunity response, the sera of the same rabbits showed great differences in their respective potencies against the typical Type III strain.

If the homologous potency be dismissed as uniformly good in all of the anti-Thomas sera, it is possible to arrange the sera from the ten rabbits into three groups in respect to their potency against typical

TABLE II.

Variations in Relative Potencies of Anti-Thomas Immune Serum from Different Rabbits in Respect to Antibodies Reactive with Typical Type III Pneumococci and Antibodies Reactive with the Thomas (Homologous) Strain.

Anti-Thomas sera	Antibodies reactive with typical Type III pneumococci					Antibodies reactive with Thomas (homologous) strain		
	Agglutination			Precipitation of S substance contained in culture filtrate	Passive protection of mice against 1×10^{-4} or 1×10^{-5} cc. of culture	Agglutination of unheated culture by 1/160 or 1/320 dilution of serum	Precipitation of S substance contained in culture filtrate in high dilution of serum or of antigen	Passive protection of mice against at least 1×10^{-7} cc. of culture
	Unheated culture	Heated culture	Concentrated suspension of heated bacterial cells					
Serum from 2 rabbits	+	+	+	+	+	+	+	+
Serum from 7 rabbits	0	±	±	0	+	+	+	+
Serum from 1 rabbit	0	0	0	0	0	+	+	+

0 = negative results in all tests with all sera; ± = definitely positive results with 5 of the sera, but equivocal results with 2 sera of this group; + = definitely positive results in all tests with all sera.

Type III pneumococci. The first group includes the sera from two rabbits: these anti-Thomas sera agglutinated unheated broth cultures of Type III pneumococci almost as well as the best Type III immune horse sera and were much more reactive than some of the diagnostic sera supplied by biological houses for routine typing. The second group includes the sera from seven rabbits: they failed to agglutinate unheated cultures of Type III strains, some of them agglu-

tinated heated cultures, most of them agglutinated concentrated suspensions of the bacterial cells and all of them passively protected mice against typical Type III pneumococci. The third group includes the serum from one rabbit which, although highly potent against the Thomas strain, failed entirely to show any type-specific immunity against typical Type III strains; the lack of protection against minimal doses, which is the most delicate criterion, being accepted as evidence of the complete lack of anti-Type III antibodies. From the standpoint of the probability of the presence of two different anti-S antibodies in the same antiserum, it is important to note that these marked variations in anti-Type III potency occurred in anti-Thomas sera which showed no significant differences in their potency against Thomas pneumococci.

It seems unlikely that these differences in the amounts of anti-Type III antibody in anti-Thomas sera containing uniformly large amounts of the anti-Thomas antibody, are due to differences in the antigen injected. All of our ten rabbits were not immunized at the same time, and it happened that the first four rabbits (injected in June and July) gave better anti-Type III responses than two rabbits immunized later (injected in September and October). We thought at that time that the poorer response of the latter rabbits might have been due to a change in the antigenic properties of the Thomas strain. In order to rule out the possibility of the repeated mouse passage of the culture having changed the Thomas strain in the direction of loss of its Type III antigenic capacity, two rabbits were later (November and December) immunized with the mouse passage strain and two other rabbits with vaccine prepared from a culture which had been in the ice box for 3 months without animal passage. The sera of all four animals gave the usual strong anti-Thomas response; and the variations in the anti-Type III response were unrelated to the mouse passage of the culture.

*Tests for the Presence of Type-Specific Anti-Type III Antibody in
Anti-Thomas Sera by Precipitation of Solutions of the
Purified Carbohydrate S Substance Derived from
Typical Type III Pneumococci.*

In preceding experiments, filtrates of young broth cultures of the Thomas strain and of typical Type III strains were employed as sources of the specific S substance elaborated by virulent type-specific pneumococci. It seemed important, however, to test the Thomas antisera against solutions of the purified carbohydrate S substance derived from typical Type III strains (4). A sample of the carbohydrate S substance furnished for this purpose by Dr. O. T. Avery

of the Hospital of The Rockefeller Institute, was tested against the anti-Thomas immune sera. The tests were made by adding 0.2 cc. of serum to 0.5 cc. of three different dilutions of the S substance (1/10,000, 1/50,000 and 1/100,000). The immune sera from four of the animals immunized earlier in the investigation were no longer available at the time the purified solution was obtained.

The results of the tests with the purified carbohydrate confirmed the results of the preceding tests with the filtrates of the Type III cultures, for the sera of some of the rabbits immunized with Thomas pneumococci precipitated the solutions of the highly specific and chemically purified carbohydrate substance prepared from typical Type III pneumococci. The prozone phenomenon was much more marked than in tests with anti-Type III horse serum. The two most reactive anti-Thomas sera precipitated the 1/50,000 solution better than the 1/10,000 solution, and gave no definite reaction at all in tests with 1/1,000 solution. This marked prozone made it seem inadvisable to attempt to increase the number of positively reacting anti-Thomas sera by repeating the tests with higher concentrations of antigen.

Absorption of Type III Immune Horse Sera with Suspensions of Typical Type III Pneumococci and with Suspensions of the Thomas Strain.

Anti-Type III serum was absorbed with suspensions of typical Type III pneumococci and with suspensions of Thomas pneumococci. In view of the factors that may influence the results of absorption tests, ten experiments were made with three different anti-Type III immune sera, under quantitatively different sets of conditions.

The results of these experiments were the same as those usually obtained in reciprocal absorption experiments with immunologically related, but different, kinds of bacteria. Absorption of the anti-Type III serum with the typical strain (homologous) completely exhausted it not only of antibodies reactive with typical Type III strains but also of those reactive with the Thomas strain. On the other hand, repeated absorption with the Thomas bacteria (heterologous) removed only the antibodies reactive with the Thomas strain and had little, if any, effect upon the potency of the serum when tested against the typical strain. The failure of repeated absorption with the Thomas bacteria to reduce the anti-Type III potency seems to us

to indicate the presence of at least two different type-specific (anti-S) antibodies in Type III immune horse serum, only one of which can be removed by the Thomas strain.

Absorption of Anti-Thomas Immune Sera with Suspensions of Typical Type III Pneumococci and with Suspensions of the Thomas Strain.

Anti-Thomas serum, potent against both typical Type III strains and the homologous Thomas strain, was absorbed with suspensions of the typical Type III bacteria and with suspensions of the Thomas bacteria under conditions analogous to those employed in the absorption of anti-Type III serum. The results of these experiments, which have been repeated many times with four different anti-Thomas immune sera, are summarized as follows:

Absorption of anti-Thomas serum with the homologous strain stripped the serum of antibodies reactive with typical Type III organisms as well as those reactive with the homologous (Thomas) organisms. Absorption with the typical Type III strain, on the other hand, removed only the antibodies reactive with typical Type III organisms and did not significantly diminish the anti-Thomas potency of the serum.

Tests with Other Typical Strains of Type III Pneumococci.

In most of the previously described experiments, one strain (A 66, Hospital of The Rockefeller Institute) was utilized as the representative of the typical Type III group. In order to determine if the relations found between the Thomas strain and the representative typical strain would hold true for other "typical" strains of Type III pneumococci we have repeated most of the described experiments with three different Type III strains recently isolated from different patients at the Vanderbilt University Hospital.

The anti-Thomas serum agglutinated and protected against the recently isolated strains as well as in the previous tests with the Rockefeller laboratory strain. Similarly, the Type III immune horse sera were completely stripped of antibodies (both anti-Type III and anti-Thomas) by absorption with the Nashville strains; and absorption of the anti-Thomas sera removed the anti-Type III and not the anti-

Thomas antibodies just as had absorption with the previously used Rockefeller strain. The repetition of the preceding experiments with these different and recently isolated strains adds considerable strength to all of the results and makes the absorption experiments much more convincing.

While the three strains (isolated in Nashville) represent too small a number to argue for the immunological homogeneity of the Type III group, the experiments with the recently isolated strains do serve to rule out the possibility that the preceding absorption results were due simply to our having used the same strain as that commonly used in the production of the Type III diagnostic serum in the different laboratories, and show that the anti-Thomas immune serum contains antibodies reactive with more than one strain of "typical" Type III pneumococci.

The necessity of repeating the preceding work with those additional strains which were known not to be the same as those utilized in the production of the anti-Type III serum is especially evident in view of the possibility that all of the biological producing laboratories may use the same strain in the production of anti-Type III diagnostic serum. And, it seemed probable to us that perhaps this one strain, the original source having been the Hospital of The Rockefeller Institute, might be the same one as that which we have employed as the representative Type III strain in our preceding experiments.

The Protection Test as a Criterion of the "Type Purity" of Pneumococcus Cultures.

The Thomas strain has been plated out repeatedly and we are convinced that its serological relationship to Type III is real, and not an apparent relationship due to the use of a mixed culture containing a few Type III organisms together with an unrelated Group IV strain. The fact that Type III immune horse sera produced in four different laboratories (by immunization with presumably "pure" Type III organisms) possessed marked protective power against the Thomas strain, seems to us to be in itself convincing evidence of the "type purity" of the culture.

As pointed out by Avery (1) specific protection is the ultimate criterion of type specificity among the pneumococci. Since protection tests offer the most delicate index of the presence of pneumococcus type-specific antibodies and suffice to detect them in antisera when test-tube methods fail, they should likewise prove to be the most delicate criteria of the "type purity" of pneumococcus cultures. For example, agglutination with the usual type sera would probably fail to detect

the presence of virulent Group IV pneumococci if mixed in small amounts with a Type III culture, but if passive protection tests were made, the mice would be infected by the small numbers of virulent Group IV organisms in spite of the protection conferred against the Type III bacteria themselves.

While it seemed certain that this principle would always hold true with virulent cultures, we have made two different sorts of experiments in order to test it. First, experiments were made in which the protective power of Type III serum was tested against: (a) Type III bacteria by themselves; (b) mixed cultures containing Type III and Type II bacteria in proportion of 1,000 to 1; (c) mixed cultures containing Type III and Type II in proportion of 10,000 to 1. The results of these experiments were exactly what one would expect if the presence of sensitized Type III pneumococci did not affect the virulence of the heterologous organism. The Type III serum protected against Type III alone, and failed against mixtures of Type III bacteria with 0.1 and 0.01 per cent of the heterologous organisms. All of the mice were autopsied. It is obvious that the experiment was concerned only with the animals which were killed by the mixed culture and not by the same amount of "pure" Type III; and typing of the heart's blood culture from these mice indicated that the heterologous organism was the sole cause of death and that the sensitized Type III bacteria had failed to survive in the blood stream even in mice having a septicemia due to the heterologous type.

Second, experiments were made to test the effect of the presence of small numbers of the Thomas bacteria upon the protective action of Type II serum; tests being made against: (a) Type II bacteria alone; (b) mixtures of Type II and Thomas bacteria in proportion of 10,000 to 1; (c) mixtures of Type II and Thomas bacteria in proportion of 100,000 to 1. The results of these experiments were analogous to the first ones; and in this case (due to the higher degree of homologous protection of the Type II serum), the protection test served to detect the presence of Thomas (heterologous) bacteria in the Type II culture even when present in the proportion of 1 to 100,000 of the homologous organisms.

DISCUSSION.

The preceding experiments dealt with the immunological properties of the Thomas strain of *Pneumococcus* which is related to but not identical with typical Type III strains. In respect to the reactions in potent anti-Type III immune horse serum, the relationship between the Thomas strain and typical Type III strains is about the same as that evidenced in anti-Type II serum between typical Type II strains and most of Avery's Subgroup II strains. But, when the comparison is made in anti-Thomas immune serum, it is evident that the relationship of the Thomas strain to typical Type III pneumococcus is different, for the anti-Thomas immune serum (from most rabbits) agglutinates and

protects against typical Type III, while none of the anti-Subgroup II immune sera were reactive against typical Type II pneumococci. The production of sera reactive against typical Type III pneumococci by injection of the Thomas strain is particularly interesting in view of the rarity of obtaining an effective anti-Type III immunity response in rabbits by the injection of typical Type III pneumococci. From these results it appears that antibodies reactive with typical Type III pneumococci can be produced more readily when rabbits are immunized with the Thomas bacteria than when immunized with typical Type III bacteria themselves. Whether the antigen in the Thomas bacterial cell which is responsible for the antibody reactive with the Type III bacteria is the same as the corresponding antigen in the Type III bacterial cell is another question.

The anti-Type III immune serum from different horses and the anti-Thomas serum from different rabbits, usually contained antibodies reactive both with the Thomas bacteria and with the typical Type III bacteria. It is particularly important that there was an entire lack of any regular relation between the relative anti-Thomas and anti-Type III potencies of the different individual antisera. The pronounced variations in the relative potencies of individual antisera in respect to antibodies reactive with the two kinds of pneumococci may represent differences in the relative proportion of two different anti-S antibodies in the antiserum from different individual animals. The variation in the anti-Type III potency of the different anti-Thomas sera is probably due to differences in the response of the individual rabbits to the particular Thomas antigen which gives rise to the anti-Type III antibody, for Tillett (3) found marked differences in the individual anti-Type III responses of rabbits when Type III bacteria themselves were injected. The possible variations in the antigenic character of the cultures (both of the Thomas and of the typical Type III strains) does not seem to be a likely explanation of the variations in the immune sera for we frequently obtained wide differences in the relative anti-Type III potency in the anti-Thomas sera of different rabbits immunized at the same time with equal amounts of the same Thomas vaccine.

The results of absorption experiments with both typical Type III antiserum and Thomas antiserum were the same as those usually ob-

tained in similar tests with immunologically related, but not identical bacteria. That is, homologous absorption removed all of the antibodies from each serum, and heterologous absorption removed only the antibodies reactive with the strain used in absorption and failed to exhaust the serum of antibodies reactive with the strain used in immunization. The failure of reciprocal absorption together with the variations in the relative potencies $\left(\text{ratio of } \frac{\text{Anti-Thomas potency}}{\text{Anti-Type III potency}} \right)$ of the antiserum from different individual animals could be presented as presumptive evidence that two different anti-S antibodies are contained in Type III immune horse serum. Although there is no evidence of complexity among the type-specific antigens of the "fixed" types of pneumococci, the same S substance united with slightly different protein constituents might give rise to related but slightly different type-specific antibodies.

In his original paper on the Subgroup Type II pneumococci, Avery (1) pointed out that the serological relationship did not in itself indicate that the Subgroup Type II and typical Type II strains were related by the lineage of common descent. The later developments in knowledge (2) of the antigenic constituents of the pneumococcus cell show more clearly that a serological relationship like that between the Thomas strain and typical Type III strains is not always a true index of phylogenetic relationship. It is now well known that there are two sorts of antigen-antibody systems involved in the immunological reactions of pneumococci: the S-anti-S reactions of type specificity, and the P-anti-P reactions of species specificity. There is much evidence that the second of these reactions is the more likely to indicate phylogenetic or truly biological relationship. Since the S-anti-S reactions separate into distinct "types" the pneumococci which manifest group relationship by P-anti-P reactions, it is important to recognize the possibility of biologically fortuitous likenesses in the chemical structure of some one of the cell constituents of phylogenetically unrelated bacteria. This possibility is well illustrated by the similarity between the S substance of Type II pneumococci and the S substance of some strains of Friedländer's bacillus (5). The common sense of the biologist would preclude the assumption of a closer phylogenetic relationship between Type II pneumococcus and Friedländer's bacil-

lus than between Type II pneumococcus and Type I or III, simply because of a greater chemical likeness between the carbohydrates elaborated by the bacteria. But, in point of fact, as far as the S-anti-S reactions are concerned, the serological relationship of the Thomas bacteria to the typical Type III bacteria is no more pronounced than that between Friedländer bacilli and Type II pneumococci; and hence, there is no real reason to believe that the Thomas strain is any more closely related, in a truly biological sense, to Type III than to any other virulent *Pneumococcus*.

In the absence of any evidence of phylogenetic relationship, the Thomas strain can best be considered as a *Pneumococcus* which, in addition to distinct immunological properties of its own, possesses a partial antigenic relationship to the typical Type III pneumococcus. The degree of type specificity manifested by Types I, II and III pneumococci is of a higher order than that usually obtained between the "types" contained in most groups of bacteria. But, in view of the wide range of immunological possibilities that are presented by Group IV pneumococci, one can expect to find a certain number of pneumococci that are related to but not identical with one of the "fixed" types. While there have been few, if any reports of pneumococci related to Type III it is quite possible that the use of a more highly reactive Type III diagnostic antiserum would result in the detection of strains related to Type III which would be included within Group IV on the basis of typing tests with weak Type III antiserum. This possibility is mentioned because of our own experience with the Thomas strain. When first typed in our laboratory, it was considered a Group IV strain and its Type III relationship was not recognized until a subsequent typing test was made with a more potent Type III antiserum than that which we had been using for routine typing.

SUMMARY.

The paper reports a study of a virulent, S-producing strain of *Pneumococcus* which is immunologically related to, but not identical with typical strains of Type III pneumococcus. In a potent anti-Type III serum, the relationship of this strain to typical Type III strains appears to be about the same as the relationship of Avery's Subgroup Type II

strains to typical Type II. But a more pronounced distinction is evident in the antiserum produced by immunization with the strain related to Type III. This antiserum contained antibodies specifically reactive with typical Type III bacteria as well as antibodies reactive with the homologous strain, while anti-Subgroup Type II immune sera are devoid of antibodies reactive with typical Type II pneumococci.

The results of absorption experiments were the same as those usually obtained with immunologically related, but not identical bacteria. The failure of reciprocal absorption and the marked variations in the relative potencies of the antiserum from different individual animals might be presented as presumptive evidence that two different anti-S antibodies are contained in Type III immune horse serum.

The theoretical significance of virulent pneumococci which are related to but not identical with the "fixed" types, is discussed from the standpoint of their importance in the biological classification of the *Pneumococcus* group.

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STUDIES ON IMMUNOLOGICAL RELATIONSHIPS AMONG THE PNEUMOCOCCI.

II. A COMPARISON OF THE ANTIBODY RESPONSES OF MICE AND OF RABBITS TO IMMUNIZATION WITH TYPICAL TYPE III PNEUMOCOCCI AND TO IMMUNIZATION WITH A RELATED STRAIN.

By ANNIE LUVERNE HARRIS,* JOHN Y. SUGG, AND JAMES M. NEILL, Ph.D.
(From the Department of Bacteriology and Immunology, Vanderbilt University
Medical School, Nashville.)

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INTRODUCTION.

The previous paper (1) dealt with the properties of a mouse-virulent strain (Thomas)¹ of *Pneumococcus*, which is immunologically related to, but not identical with typical Type III pneumococci. One of its most prominent properties is its antigenic effectiveness in rabbits. In contrast to the rarity of a detectable type-specific (anti-S)² response on injection of typical Type III strains, immunization with the Thomas strain invariably yielded high titres of anti-Thomas antibodies and usually also yielded measurable amounts of antibodies specifically² reactive with Type III pneumococci. These differences between the

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¹ The terms "Thomas" strain and "anti-Thomas" serum are employed, as in the preceding paper, to refer to the "non-typical" strain and its antiserum.

² The distinctions between the anti-S antibody and the anti-P antibody in the serological reactions of Type III pneumococci have been pointed out in detail by Tillett (3). As stated in the preceding paper, the immunity systems involved in the reactions of anti-Thomas serum with typical Type III pneumococci are the same or similar to the S-anti-S systems involved in the type-specific reactions of the "fixed" types with their homologous antisera. The anti-Thomas serum is just as devoid of effect upon Types I and II pneumococci as is anti-Type III serum, and the Thomas bacteria are just as little affected by anti-Type I or anti-Type II serum as are typical Type III pneumococci.

response of rabbits to the Thomas strain and their response to typical Type III strains seem to be important, especially in regard to the anti-Type III response elicited by injection of the Thomas strain.

It has been general experience (2), not only with rabbits but also with larger animals, that potent anti-Type III immune sera are more difficult to obtain than are potent anti-Type I and anti-Type II immune sera. The question arises, therefore, whether the antigen which gives rise to the Type III anti-S antibody is intrinsically a less effective antigen in all animals, or whether this antigen which seems to be so ineffective might not in other animals elicit responses comparable to those invoked by other types of pneumococcus anti-S antigens.

As a step in the accumulation of data on this question, we have compared the antibody response invoked in rabbits with that invoked in mice when the two species of animals are immunized with typical Type III pneumococci and with the related, but different, Thomas strain. The measurements of the immunity response have included antibodies reactive with the Thomas strain as well as those reactive with typical Type III strains, for anti-Type III horse serum and anti-Thomas rabbit serum usually contain antibodies reactive with both kinds of pneumococci (Thomas and Type III).

EXPERIMENTAL.

Comparison of the Antibody Response of Rabbits to Immunization with Typical Type III Pneumococci with their Response to Immunization with the Thomas Strain When the Amounts of Bacterial Antigen Are Equal.

The fact that rabbits respond poorly to Type III pneumococci is clearly shown in Tillett's (3) extensive study in which 18 out of 28 rabbits failed to produce in their serum even the small amount of type-specific antibody detectable by the delicate mouse protection test. That the Thomas strain is more effective is evident from the results of the preceding investigation (1) in which, in addition to the uniformly high degree of anti-Thomas potency in all sera, only 1 out of 10 rabbits failed to produce the small amount of antibody required to specifically protect mice against virulent Type III pneumococci. These facts strongly suggest that the Thomas bacteria are more effec-

tive in invoking an anti-Type III response in rabbits than are the typical Type III bacteria themselves. It seemed desirable, however,

TABLE I.

Comparison of the Response of Rabbits to the Injection of Typical Type III Pneumococci with the Response to Equal Amounts of the Thomas Strain.

Immune serum		Anti-Type III potency									
Rabbit	Immunization material	Agglutination						Passive protection of mice <i>vs.</i> virulent Type III culture			
		Heated broth culture			Concentrated suspension of heated bacterial cells						
		Serum dilution			Serum dilution			Dose of culture, cc.			
		Undiluted	2/5	1/5	Undiluted	1/5	1/10	1 × 10 ⁻³	1 × 10 ⁻⁴	1 × 10 ⁻⁵	1 × 10 ⁻⁶
1	Thomas strain	++	+	0	fff	ff	f	D	S	S	S
2	Thomas strain	0	0	0	f	0	0	D	D-S	S	S
3	Typical Type III strain	0	0	0	0	0	0	D	D	D	D
4	Typical Type III strain	0	0	0	0	0	0	D	D	D	D
Anti-Type III horse serum (control)		++++	+++	+++	fff	fff	fff	D-S	S	S	S
Anti-Type I and anti-Type II horse serum (controls)		0	0	0	0	0	0	D	D	D	D

+++ = compact disc, with clear supernatant, after 2 hours at 37°C.; ++ = compact disc, but supernatant not entirely clear; + = definite granulation of the bacteria which persists after thorough shaking.

fff = sedimentation of agglutinated particles almost complete within 10 minutes at 25°C.; ff = sedimentation begun and fluid partially cleared by aggregation of agglutinated particles; f = definite granulation of the bacteria but fluid not cleared by aggregation.

D = mice died within 72 hours in all tests; D-S = protection irregular, mice dying in some tests and surviving in others; S = mice survived in all tests. Virulence controls died within 72 hours when injected with 1×10^{-3} cc. of culture.

that a comparison be made by experiments in which the amount of bacterial antigen and the number of injections were exactly the same.

Two rabbits were immunized against typical Type III pneumococci by two courses of six daily injections of heat-killed bacterial suspension equivalent to 5 cc.

of broth culture in each dose; a week's rest was given between the first and second courses, and the animals were bled 10 days after the last injection. Two other rabbits of the same weight were immunized with the Thomas strain by the same number of injections and with the same amount of bacterial suspension. A summary of the tests of the immune sera is presented in Table I.

The results in Table I show that the particular rabbits injected with typical Type III pneumococci in this experiment failed to produce antibodies reactive with the homologous bacteria, although both of the rabbits immunized with the same amount of the related but different strain, produced antibodies reactive with typical Type III pneumococci. While the anti-Type III potency of the anti-Type III and anti-Thomas serum as obtained from different immune rabbits will vary, the individual rabbits of each series used in this particular experiment yielded serum that can be accepted as possessing about the average potency exhibited by Tillett's series of 28 rabbits and by our previous series of 10 rabbits. Hence, with the above results considered as representative of those most likely to be obtained with rabbits, it is evident that rabbits can produce antibodies specifically reactive with typical Type III pneumococci more readily when injected with the Thomas strain than when injected with typical Type III pneumococci themselves. The results in Table I are important as evidence that this relation holds true when the amount of total bacterial antigen and the conditions of immunization are kept constant.

Both of the rabbits injected with the Thomas bacteria yielded serum potent against the homologous strain, while neither of the anti-Type III rabbit sera reacted at all with the Thomas strain. Since our chief interest is in the anti-Type III response, the results of the tests of the anti-Thomas potency are not included in Table I.

Comparison of the Active Immunity Response of Mice to Vaccination with Typical Type III Pneumococci with the Response to Vaccination with the Thomas Strain of Pneumococci.

The preceding experiment dealt with the responses of rabbits to immunization with typical Type III pneumococci and to immunization with the Thomas strain. The following experiment deals with the responses of mice to immunization with these two related but different kinds of pneumococci.

Immunization.—Two series of 60 white mice, about 3 months old, were selected for immunization; one series was injected with the Thomas strain and the other with a typical Type III strain. The organisms from 10 hour broth cultures were resuspended in salt solution and heated at 60°C. for 30 minutes. The suspensions of the two kinds of pneumococci were compared and when not approximately equal in turbidity, the stronger suspension was diluted until the concentration of total bacterial substance in each was about the same. Each mouse received, subcutaneously, 0.5 cc. of the respective vaccine (Type III or Thomas), equivalent to 0.15 cc. of broth culture, every 2 days for six doses; after which time one dose was given intraperitoneally. 10 days after the last injection, 20 mice from each series were tested for immunity against the two kinds of pneumococci. The remainder were given a second course of immunization with freshly prepared vaccine in the same doses as in the first course. After a rest period of 10 days, another lot of the immunized mice was tested for immunity either against the Thomas strain or against the typical Type III strain.

Tests of Immunity.—The active immunity of the vaccinated mice was tested by intraperitoneal injection of 0.5 cc. of broth containing the desired amount of a 10 hour broth culture of virulent pneumococci. Some of the mice vaccinated with typical Type III were tested against the Thomas strain and others against the homologous typical Type III strain; the Thomas-vaccinated mice were likewise tested against the typical Type III strain as well as against the homologous strain.

The doses used were 1×10^{-5} and 1×10^{-6} cc. of broth culture. These doses are not unreasonably small since both cultures were highly virulent and killed mice regularly in doses of 1×10^{-3} cc. Moreover, it seemed unwise to overtax the active immunity of the mice, especially in the case of Type III, in which the passive immunity conferred by immune horse serum is usually overwhelmed by doses above 1×10^{-4} cc. As a matter of fact, however, the Type III-vaccinated mice which had responded at all, apparently were able to resist doses approximately equivalent to those against which Type III horse serum can protect, for 3 out of 4 vaccinated mice which were tested against 1×10^{-4} cc. resisted this large dose in a later experiment.

The tests were made after both one and two courses of vaccinations. However, one course proved as effective as two courses, and since there is no essential difference in the tests, the results of the entire experiment are summarized in Table II.

Table II presents a summary of the results of experiments upon the active immunity response of mice to vaccination with the two related, but different, kinds of pneumococci—*i.e.*, a typical Type III strain and the Thomas strain. In a previous investigation (4) on the active immunization of mice against Type II pneumococci, it was found that within the zone of reasonably small dosage, the number of invading bacteria was a relatively unimportant factor in com-

parison to the factor of the differences in the immunity responses of the individual mice. For this reason, we believe the results of experiments on the active immunity response of mice can best be analyzed upon the basis of the percentage of individuals protected among a large group of vaccinated mice.

In the analysis of Table II, it is best to consider first the response of the mice as indicated by their immunity against the same strain as that with which they were vaccinated. It is evident that the mice vaccinated with Type III pneumococci responded fairly well in respect to the development of an immunity affording homologous protection, for 75 per cent of the 20 animals tested survived the injection

TABLE II.

Summary of Tests of the Response of Mice to Immunization with the Typical Type III Strain and with the Thomas Strain as Evidenced by Their Active Immunity against Virulent Cultures of These Two Kinds of Pneumococci.

Vaccinated mice	Mice vaccinated with typical Type III pneumococci		Mice vaccinated with Thomas strain of pneumococci	
	Homologous protection (vs. 1×10^{-3} or 1×10^{-6} cc. of virulent Type III culture)	Heterologous protection (vs. 1×10^{-3} or 1×10^{-6} cc. of virulent Thomas culture)	Homologous protection (vs. 1×10^{-3} or 1×10^{-6} cc. of virulent Thomas culture)	Heterologous protection (vs. 1×10^{-3} or 1×10^{-6} cc. of virulent Type III culture)
Number tested.....	20	15	22	15
Number protected.....	15	0	9	0
Percentage of protection.....	75	0	40	0

of virulent Type III bacteria. The response of the mice to the Thomas vaccination was apparently somewhat less effective, for only 40 per cent of the animals tested survived the injection of the Thomas or homologous bacteria. Thus, it is evident that from the standpoint of the development of immunity against the homologous kind of pneumococci, mice differ from rabbits in respect to the relative effectiveness of the typical Type III antigen and the Thomas antigen. Rabbits almost invariably respond well to the Thomas strain by producing immune sera of a high degree of potency, comparable in all respects to that elicited by Type II pneumococci, while an effective response of rabbits to the typical Type III antigen is the excep-

tion rather than the rule. The important fact in regard to the homologous protection in Table II is not that the mice do not respond uniformly well to the Thomas vaccination, but rather that such a large percentage of the mice are effectively protected by the Type III vaccination. The percentage of homologous protection exhibited by the Thomas-vaccinated group is just as high as that obtained in a previous investigation with Type II pneumococci, in which only 30 to 40 per cent of a large number of mice were effectively protected by homologous vaccination by the procedure employed in the present study. Thus, it appears that in respect to the development of homologous protection, mice respond just as well, and even better, to the Type III antigen than they do to two other kinds of pneumococci which in rabbits invoke incomparably better responses than do Type III pneumococci.

The response of the mice as evidenced by their immunity against the heterologous strain is less important, although it is interesting to observe that the mice failed to develop any detectable immunity against the related strain. If the mice had been more uniformly protected against the homologous strain, the lack of immunity against the heterologous strain would be important, for Type III immunization of horses does give antibodies reactive with the Thomas strain, and Thomas immunization of rabbits usually gives antibodies reactive with typical Type III strains.

Immunity Response of Mice as Evidenced by Passive Protection Experiments.

Since active immunity is not always the same as passive immunity, it seemed important to determine whether or not the serum of the vaccinated mice could confer passive protection to other mice.

In order to eliminate the factor of differences in the individual responses of the vaccinated mice, the serum from 8 mice vaccinated with Thomas bacteria was pooled and used as the anti-Thomas immune mouse serum; and the serum from 8 mice vaccinated with the Type III bacteria was used collectively as the anti-Type III immune mouse serum. Passive protection experiments were then made by the usual procedure with doses of 1×10^{-5} and 1×10^{-6} cc. of culture, the anti-Thomas serum being tested against both Thomas and Type III bacteria and the anti-Type III serum against both Type III and Thomas bacteria.

The results of the passive protection experiments were in all respects analogous to those obtained in the previously described active immunity tests. The factor of differences in the individual responses of the vaccinated mice was eliminated by pooling the immune serum. The anti-Type III immune mouse serum gave passive protection against Type III alone and not against the related Thomas bacteria; and the anti-Thomas immune serum gave protection against the Thomas bacteria and not against the typical Type III strain. The immune mouse sera, however, are scarcely comparable in homologous potency to the anti-Type III horse serum or the anti-Thomas rabbit serum; and hence their lack of heterologous potency is of less importance, for it is common experience that an antiserum of a slight degree of homologous potency is more usually of a higher degree of specificity.

Tests with a Recently Isolated Strain of Typical Type III Pneumococcus of Different Origin from the Type III Strain Employed in Vaccination of the Mice.

In the preceding experiments, the mice were tested against the same strain of typical Type III pneumococci as that employed in the vaccination. In the previous investigation (1), the immunological relationships evidenced in rabbit and horse antisera between this strain of Type III pneumococci and the Thomas strain were proved to hold true for three other strains of Type III pneumococci which had been recently isolated in Nashville. While there was this evidence in favor of the immunological homogeneity of typical Type III strains, it seemed important to repeat the experiments with a Type III strain known to be of different origin from the Rockefeller strain with which the mice had been vaccinated. A strain of Type III pneumococci which had been isolated about 10 days previously from a patient in the Vanderbilt University Hospital was utilized for this purpose in active immunity tests against mice vaccinated with the Rockefeller Type III strain and against mice vaccinated with the Thomas strain. Passive protection tests were also made as described previously with both anti-Type III and anti-Thomas immune mouse serum.

The results were exactly the same as those reported for the tests with the strain which had been used in vaccination. The mice vac-

minated with the Rockefeller Type III strain were protected and the mice vaccinated with the Thomas strain were not protected against the Nashville Type III strain. Similarly, the immune serum from mice vaccinated with the Rockefeller Type III strain did give, and the serum from the mice vaccinated with the Thomas strain did not give passive protection to other mice against the Nashville Type III strain.

These tests were made against only the one foreign strain and are not presented as an argument for the homogeneity of the Type III group of pneumococci. But the identical results obtained in these experiments together with those in the preceding investigation, prove that the failure of reciprocal protection of mice against Type III pneumococci by active or passive immunization with the Thomas strain, and the failure of reciprocal protection of mice against the Thomas strain by active or passive immunization with the Rockefeller typical Type III strain, are not phenomena dependent upon the use of precisely the same Type III strain in the immunization and subsequent immunity tests.

Virulence of the Thomas Strain and of the Typical Type III Strain for Rabbits.

In view of the marked differences between the relative immunity responses of mice and those of rabbits to immunization with typical Type III strains and the Thomas strain, it is important to record the tests of the virulence of these strains for the two species of animals. Both the Thomas strain and the typical Type III strain (A 66, Hospital of The Rockefeller Institute) were highly virulent for mice, killing them regularly when doses of 1×10^{-8} cc. of broth culture were injected; but it has been shown by Tillett (5) that the virulence of Type III pneumococci for mice is by no means an index of the virulence of the same strains for rabbits.

Because of the probably marked differences in the natural immunity of different individual rabbits which has been suggested by Tillett, four rabbits were tested with each strain. The individual doses were 1 cc. intravenously, 5 cc. intravenously, 5 cc. intraperitoneally and 10 cc. intraperitoneally. None of the rabbits died even from the largest doses of the typical Type III strain. Two of the rabbits (those injected with 5 cc. and with 10 cc. intraperitoneally) were killed by the Thomas strain. The cultures injected had not been passed through rabbits.

From these results, it is evident that, although neither strain is highly virulent (at least without exaltation of potential virulence by rabbit passage), the Thomas strain is more virulent for rabbits than is the typical Type III strain.

DISCUSSION.

The preceding experiments have dealt with the relative effectiveness of the responses of mice and of rabbits to immunization with a typical Type III strain in comparison with their response to immunization with the Thomas strain which is related to but not identical with Type III pneumococcus.

In regard to the response of rabbits, the apparent relationship between the antigenic effectiveness of the two kinds of pneumococci was the same as that already described in the preceding paper; but the results of the present study are important in that the conditions of immunization and the dosage were kept constant in the animals injected with the two strains. From the standpoint of the development of homologous, specific antibodies, the Thomas strain was incomparably more effective; the anti-Thomas rabbit sera having, as usual, a high degree of anti-Thomas potency; and the anti-Type III rabbit sera being, as is frequently the case, devoid of anti-Type III potency. The greater effectiveness in rabbits of the Thomas antigen was also true from the standpoint of anti-Type III potency; and the results in this investigation, as in the preceding one, furnish evidence that rabbits can produce antibodies specifically reactive with typical Type III pneumococci, more readily when injected with the Thomas strain than when injected with typical Type III pneumococci themselves.

With mice, the results were quite different. In the experiments with these animals, it was found that vaccination with typical Type III pneumococci elicited somewhat better immunity responses,—as indicated by the higher percentage of individuals which were protected,—than did vaccination with the Thomas strain. The immunity in both instances was limited to homologous protection, for both the Thomas strain (which in rabbits invoked an anti-Type III response) and the typical Type III strain (which in horses invokes an anti-Thomas response) failed to invoke an immunity against the related but different kind of *Pneumococcus*. From the standpoint of the

active immunity exemplified by homologous protection, the typical Type III strain proved not only to be more effective antigenically than was the Thomas strain, but also, in comparison with the results of a previous study (4), it appeared to be a more effective antigen in mice than are Type II pneumococci,—although the latter, like the Thomas pneumococci, almost invariably give better immunity responses in rabbits.

Thus, the results show that the relative antigenic effectiveness of these two kinds of related pneumococci, is different in different animals; in rabbits, the Thomas strain is more effective than typical Type III; in mice, the typical Type III is better than Thomas. These relationships furnish an example of the influence of the species of animal upon the apparent effectiveness of bacterial antigens, for while the potential antigenicity is resident in the chemical structure of the antigen, its actual effectiveness in practice is determined by the individual response of the particular animal. This example of the marked differences in the selective effectiveness of the two different antigens is the more interesting in view of the close serological relationship of the two kinds of pneumococci utilized as antigens.

SUMMARY.

The paper reports an experimental comparison of the antibody responses of mice and of rabbits to immunization with typical Type III pneumococci and a strain related to Type III. The results as a whole show that the relative antigenic effectiveness of these two kinds of related pneumococci, is different in different animals. In rabbits the strain related to but not identical with Type III elicits the better response; in mice, the typical Type III strain is more effective. These relationships furnish an example of the influence of the species of animal upon the effectiveness of even closely related bacterial antigens.

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UDDER INFECTION WITH STREPTOCOCCI OF THE SCARLET FEVER TYPE.

I. SPONTANEOUS AND EXPERIMENTAL UDDER INFECTION.

BY F. S. JONES, V.M.D., AND RALPH B. LITTLE, V.M.D.

(From the Department of Animal Pathology of The Rockefeller Institute for Medical Research, Princeton, N. J.)

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During the past four decades many outbreaks of scarlet fever attributed to contamination of milk supplies have been reported. This occurrence claimed considerable attention during the later '80's of the 19th century. Since then, although outbreaks attributed to milk have been recorded, the number has apparently declined. In certain particulars epidemics due to contaminated milk resemble each other. Their explosive nature—the bulk of the cases occur during a short period, usually within a week—is characteristic. The history that the sick have partaken of milk from a common source and the fact that the disease is not epidemic in other parts of the community having a different milk supply are equally significant.

The origin of milk-borne epidemics of scarlet fever has in the past led to much discussion.

Probably the most commonly held view is that of actual contamination of the milk by convalescents or persons actually sick with the disease. This view is supported by many recorded outbreaks in which persons known to have come in contact with scarlet fever or who were actually suffering from the disease milked the cows, mixed, handled, or bottled the milk. Among others, Hemenway (1) records a large epidemic attributed to milk thus contaminated. Other outbreaks regarded as milk-borne could be traced to actual cases who delivered the milk from door to door. Chalmers (2) reports such an outbreak. In addition the return of milk bottles from houses where scarlet fever was present was held responsible for the disease along particular milk routes.

The widespread and severe nature of certain outbreaks in England during the period from 1880 to 1900 led to investigations that directed attention to the cow as a source of infection. The difficulty of tracing human contamination strength-

ened the suspicion. Power (3) and Klein (4) as early as 1882 attributed one outbreak to the contamination of milk from a case of puerperal fever in a cow. Klein showed that cows inoculated subcutaneously with material obtained from the throats of human patients developed abscesses at the injection sites. Purulent material from such abscesses when injected into healthy cows also produced abscesses. Later Power (5), Cameron (6), and Klein (7) reported their findings in the Hendon outbreak in which they failed to find a human source of infection. Suspicion was directed to certain newly purchased cows suffering with a malady of the skin of the udder and teats. This they regarded as a specific infection transmitted by the hands of the milker. The condition was characterized by general constitutional disturbance, sore throat, discharge from the eyes and nose, vesicular eruptions of the skin of the buttocks and udder. They regarded the rupture of the teat vesicles as the source of the milk inoculation. Russell (8) encountered a similar outbreak in which 101 persons contracted scarlet fever; a malady similar to the Hendon disease affecting two cows was noted in the herd. A calf fed the milk of one of these developed a severe febrile reaction. Hill (9) likewise reported milk-borne scarlet fever; certain cows in the dairy from which the supply was drawn were found affected with Hendon disease. Hamar and Jones (10) also cited an outbreak in which a disease of the skin of cows similar to that noted by Power, Klein, and Cameron was present in the herd. They were inclined to attribute the human infection to the cows since the milk was known to be infective before human cases occurred on the farm. M'Fadyean (11) disagreed with them on the grounds that Hendon disease as a scarlatinal infection of cows is unproved and that the milker in whose family scarlet fever occurred may have been responsible for the epidemic through contamination. As a further criticism that Hendon disease is not a specific scarlatinal infection of cows he points out that although the disease was first recognized in 1882 and seemed to prevail for a year or two it had not attracted further attention until 1909 when a similar disease was reported.

It is apparent then that there are several views regarding the method by which milk may become infective: first, contamination of the milk during milking or handling; second, the return of contaminated bottles or utensils from the household in which the disease exists; and third, a scarlatinal disease of the skin and udder of cows from which discharges may enter the milk during milking.

With the change in the status of the streptococcus as the etiological agent of scarlet fever during the past few years, a fourth means of contamination becomes apparent. The findings of Savage (12), T. Smith and J. H. Brown (13), Davis and Capps (14), Brown and Orcutt (15), and Benson and Sears (16) in milk-borne epidemics of septic sore throat are of considerable interest in this connection. It

has been shown that *Streptococcus epidemicus* may be implanted in the udder and be shed in the milk in such numbers as to give rise to severe outbreaks of sore throat among those consuming the milk. That such is not unlikely in outbreaks of scarlet fever attributed to milk seems plausible. This is especially significant in many epidemics traced to dairies where no mention is made of clinical examination of the udder or bacteriological examination of the milk. The possibility of udder infection may have been overlooked.

Our problem concerns itself with udder infections with streptococci similar to those found in scarlet fever.

History of a Milk-Borne Epidemic of Scarlet Fever.

Through the courtesy of the New Jersey State Department of Health we learned of a sharp outbreak of scarlet fever in a small town. About 200 cases occurred. Of these 159 developed from May 20 to 25, 1927. The State authorities found that the bulk of the cases was confined to a certain milk route supplied by one distributor. Further information directed their attention to one of the farms supplying the dairy. On this farm a daughter had scarlet fever in March, 1927. In addition a young man employed as milker had visited his home shortly before the outbreak where there was a child sick of scarlet fever. Representatives of the State Department of Health made throat cultures from everyone suspected of contaminating the milk and obtained hemolytic streptococci in two instances from throats of men handling the milk at the distributor's. For these cultures we are indebted to Mr. J. V. Mulcahy, Chief of the Bureau of Bacteriology of the State Health Department. In addition he furnished us with a culture from the young man who milked the cows, but it was of the *viridans* type.

On May 25, Dr. I. H. Shaw, veterinarian for the State Department of Health, visited the farm and examined microscopically the milk sediment from each cow. He noted one chronic case of mastitis (Cow 11) and an acute injury of the teat of the left hind quarter (Cow 3). The milk sediment of Cow 3 contained leucocytes and cocci.

In the meantime the milk from this farm had been excluded from the supply and a pasteurizer installed in the distributor's with the

result that the epidemic rapidly subsided. At our suggestion, and through the courtesy of the State Department of Health, the farm was visited on June 4, 1927. Samples were drawn directly from the udder of each cow into separate sterile bottles.

Examination of Milk from Each Cow.

The samples were obtained on the evening of June 4, refrigerated at once, and plated late the same night. The normal appearing milk was plated in two dilutions, 1:20 and 1:100; that from abnormal quarters at 1:1,000 as well as the lower dilutions. All plates were prepared with 0.5 cc. of defibrinated horse blood and 10 or 12 cc. of melted agar. Thirteen cows comprised the herd. The udders of eleven were normal. The plate cultures revealed nothing of significance. Cow 11 had chronic mastitis of the left hind and right fore quarters. The plate cultures revealed non-hemolytic streptococci in enormous numbers both in the involved and apparently normal quarters. Cow 3 had a severe involvement of the left hind quarter. The teat had been injured, the quarter was swollen and could be milked with difficulty. The milk was yellow and of the consistency of heavy cream. All the blood in the plate containing as little as 1:1,000 cc. of milk was hemolyzed within 12 hours. The centrifuged sample revealed a large quantity of sediment consisting of packed masses of leucocytes and enormous numbers of short chained streptococci. After refrigeration for 12 hours the milk was further diluted and plated so that finally it was possible to estimate that it contained 345,000,000 streptococci per cc. Colonies sufficiently isolated were chosen for subculture. It may be said that the initial tests, such as those for the presence of capsules, the laking of blood in the test-tube, pathogenicity for rabbits, and a final pH of 5.0 in dextrose broth, indicated that this culture was of human origin. In addition to the human type a small proportion of the non-hemolytic bovine streptococci was found in the original sample.

The Spontaneous and Experimental Infection in the Cow.

Cow 3 was purchased by this Department and is hereafter referred to as No. 1452. 4 days after the first observation the quarter was swollen, firm, and the teat showed a healing scar. Yellow, purulent milk could be expressed only with difficulty. Bacteriological examination revealed relatively few hemolytic streptococci and enormous numbers of the non-hemolytic mastitis type. Evidently the bovine type noted June 4 had nearly replaced the hemolytic streptococcus. The inflammation continued in the quarter until the cow was slaughtered on June 23. During this time hemolytic streptococci were always present in small numbers and the mastitis type in enormous numbers.

On one occasion a single colony of the hemolytic type was obtained from the milk of the right hind quarter. This strain was identical in all respects with that obtained from the left hind quarter. Although the milk from the other quarters was plated frequently streptococci were not found.

On June 9, the right hind quarter was inoculated by means of a teat tube with 1/1,000,000 cc. of a 24 hour broth culture of the hemolytic streptococcus from the left hind quarter. No reaction occurred and examination of the milk during the next 3 days failed to show the organism.

On June 14, the right fore quarter was inoculated by means of a teat tube with 1/500,000 cc. of the hemolytic culture and the right hind quarter with 1/100,000 cc. of the same culture. 7 hours later the streptococcus could not be cultivated from the milk from either quarter. After 24 hours there was little to be detected clinically. The appearance of the milk was not greatly altered, and the quantity of sediment was not excessive but it contained leucocytes and a few diplococci. Plate cultures, however, revealed 25,600 hemolytic streptococci per cc. in the milk from the right hind quarter and over 1,000,000 per cc. in that from the right fore quarter. After 48 hours the right hind quarter was hot, the milk was yellow and contained large, irregular floccules. The right fore quarter revealed nothing abnormal except that the milk was yellow and flocculent. Samples from both quarters revealed an excessive amount of sediment composed of leucocytes and streptococci. The bacterial counts of the milk were: right fore quarter = 45,000,000 hemolytic streptococci per cc., and right hind quarter = 1,240,000 per cc. The next day 38,000,000 hemolytic streptococci per cc. were found in the milk from the right fore quarter and 1,240,000 per cc. in that from the right hind quarter. From this time onward the number began to decline until on the 14th day the milk from the right fore quarter failed to show streptococci while that from the right hind quarter revealed 26,000 per cc.

During the period of observation it can be said that the inoculation failed to produce well defined clinical disturbances in the inoculated quarters, although the milk was purulent.

The udder obtained from the abattoir at the time of slaughter revealed a pronounced atrophy of the left hind quarter characterized by severe degeneration of the secreting structures and hyperplasia of the interstitial tissue. The two inoculated quarters showed lesions of the mucosa of the lower portion of the udder involving the mucosa of the large milk ducts accompanied by a purulent exudate.

Since the spontaneous case of infection was complicated by injury and secondary infection with the usual type of mastitis streptococcus,

the question might be raised as to whether the factor of injury is a necessary precursor of infection. Further the experimental disease incited by inoculation failed to resemble the spontaneous disease. In order to throw more light on these questions a second series of inoculations was made.

Cow 1462.—A Holstein cow was injected by means of a teat tube inserted into the left hind quarter with 1/1,000,000 cc. of an 18 hour serum broth culture of the 6th transfer of the streptococcus obtained from the left hind quarter of Cow 1452. Plate cultures prepared from similar dilutions indicated that between 15 and 20 streptococci were injected. Plate cultures of the milk 7 hours after injection failed to show streptococci. 24 hours after injection the quarter still appeared normal. The milk contained only a little sediment composed of a few leucocytes and round cells and a few diplococci. The plate culture revealed 115,000 hemolytic streptococci per cc. After 31 hours the quarter was swollen, tense, hot, and

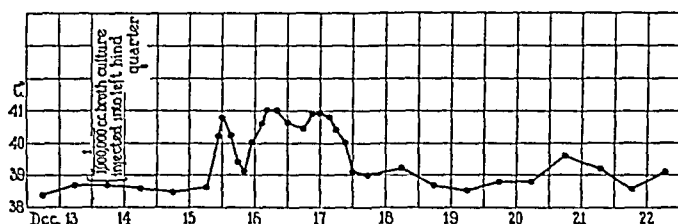


CHART 1. Temperature reaction, Cow 1462, following injection of left hind quarter.

painful. The milk contained an excess of fat but the amount of sediment was not excessive. There were 2,000,000 streptococci per cc. At 36 hours the quarter was greatly swollen. The milk was yellow and serous and contained large flocules. It coagulated on boiling. Plates revealed 8,320,000 streptococci per cc. 48 hours after injection there was a severe systemic reaction characterized by chills, fever (Chart 1), and depression. Swelling of the quarter was pronounced. The milk was seropurulent. It was estimated that 1,200,000,000 streptococci per cc. were being eliminated. On the 3rd day the quarter was distinctly reddened, the milk purulent, and 510,000 streptococci per cc. were recorded. A blood culture was negative. The cow had a fever. The reddening of the skin spread to the other quarters on the 4th day, persisted throughout the 5th day, and began to subside on the 6th day. It had disappeared by the 7th day. During this period the number of streptococci decreased until a minimum of 20,000 per cc. was reached. However they increased during the 7th, 8th, and 9th days, reaching the high point of 69,000,000 on the 8th day. There was a corresponding rise of temperature on the 7th and 8th days (Chart 1). By the 13th day their number

had declined to 2,000,000. From this time the acute inflammation gradually subsided with gradual atrophy and with a further decline in the secretion until on the 47th day only 25 or 30 cc. of purulent milk was obtained. Hemolytic streptococci were still present on this day. Chart 2 illustrates the number of streptococci per cc. of milk from the left hind quarter of Cow 1462 for 9 days following the inoculation.

It is to be noted that during the height of the attack the general reaction was severe, milk secretion was almost entirely suppressed, a fall from 8 pounds per milking to less than 1 pound being recorded.

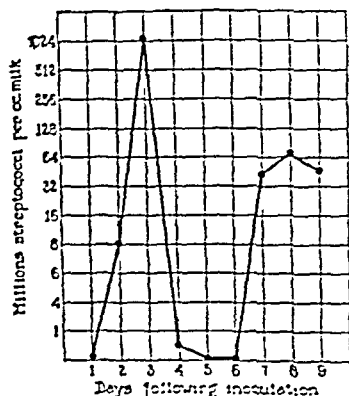


CHART 2.

CHART 2. Streptococci per cc. of milk during the first 9 days subsequent to injection of left hind quarter of Cow 1462.

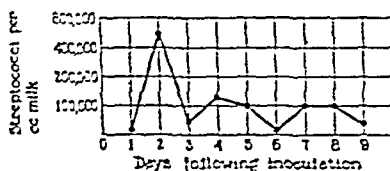


CHART 3.

CHART 3. Streptococci per cc. of milk during the first 9 days after injection of right hind quarter of Cow 1462.

27 days after the left hind quarter had been artificially infected one-millionth cc. of broth culture was instilled into the lower cistern of the right hind quarter. It was estimated that approximately 70 streptococci were injected. 7 hours later there was nothing in the appearance of the milk to arouse suspicion and 1 cc. failed to reveal the streptococcus. After 24 hours the milk appeared normal. The sediment contained a few leucocytes and plate cultures revealed 1,800 streptococci per cc. 2 days following the inoculation the quarter failed to show abnormalities. The milk was yellow and thickened; when centrifuged the sediment comprised about 1/20 of the total volume. It was composed of masses of leucocytes, diplococci, and short chained streptococci. The maximum count of 556,000 streptococci per cc. was reached on this day. During the next 9 days the udder and milk were examined daily. Lesions of the udder were not detected

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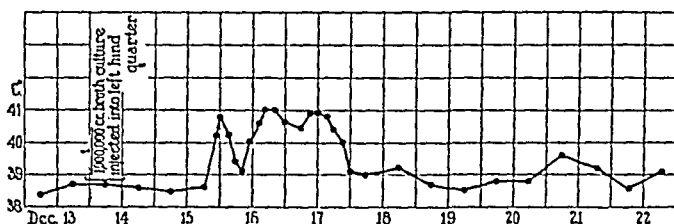


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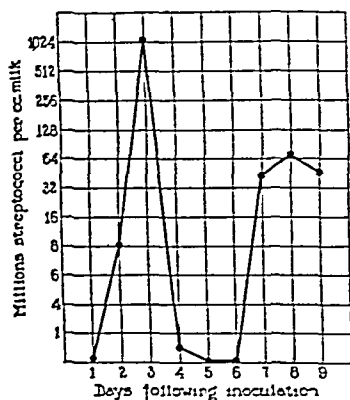


CHART 2.

CHART 2. Streptococci per cc. of milk during the first 9 days subsequent to injection of left hind quarter of Cow 1462.

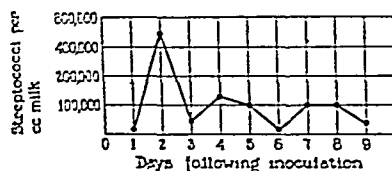


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clinically. The milk was always purulent and the sediment at times made up 1/10 of the volume. The number of streptococci varied from day to day, the minimum count recorded during this period being 18,000 and the maximum 136,000 per cc. On the 21st day the milk was still purulent and flocculent and contained 20,000 streptococci per cc. Chart 3 is included for comparison with Chart 2. It indicates the number of hemolytic streptococci eliminated per cc. during the first 9 days following inoculation of the right hind quarter. The two charts afford a sharp numerical contrast. In the primary inoculation the maximum of over 1 billion was reached on the 3rd day following injection, with a rapid fall until the 6th day and a subsequent sharp rise on the 7th, 8th, and 9th days. Chart 3 illustrates the elimination of streptococci subsequent to injection of the right hind quarter. Here the peak of 550,000 was reached on the 2nd day with a sharp decline on the 3rd day and irregularity thereafter.

It is true then that the streptococcus isolated from the spontaneous infection was of sufficient virulence in spite of cultivation for 6 months on artificial media to incite a severe mastitis. During the acute inflammation there were marked systemic disturbances characterized by fever, increased pulse and respiratory rates, congestion of the conjunctiva, inappetence, suppression of milk, and stiffness of the joints. We regarded the animal as critically ill during this period.

In data from both the spontaneously infected and the experimental cow evidence exists that the primary attack so increased the general resistance that the secondary injection resulted in entirely different manifestations. Here only mild local disease resulted; although the streptococcus established itself, its multiplication never reached the maximum recorded in the primary attack.

It may be argued that the spontaneous case (No. 1452) referred to is an isolated example of such infection.* While this is true to a certain

* While these data were being assembled, Mr. Friend Lee Mickle, Director of the Bureau of Laboratories, Connecticut State Department of Health, sent three cultures of streptococci for examination. The interest of the Health Department had been aroused because of an outbreak of mild scarlet fever which appeared among the customers along a certain milk route. One of the cultures was isolated from the udder of a cow in the herd supplying the milk, another from the throat of the owner of the herd, and a third from a case of scarlet fever on the milk route. These proved similar in cultural characters, pathogenic properties, and antigenic affinities to those obtained by us from the udders of two cows. In all probability the particulars of this outbreak will be published by the Connecticut State Department of Health.

extent, nevertheless material obtained from another case of mastitis associated with a similar organism suggests that such infections may occur at any time. Although at the time of isolation the significance of the bacteriological findings in this spontaneous case was not realized, nevertheless the organism was regarded as a human type other than *S. epidemicus*. A brief statement concerning this case follows.

Cow 4262.—Milk drawn from all four quarters into a sterile bottle May 11, 1925. When plated it was found to contain 720 colonies per cc.; 25 per cent were large zoned, hemolytic colonies. May 19, 1925, mastitis of the right fore quarter was noted. The milk was purulent and flocculent. 77,000 hemolytic colonies per cc. were recorded. On May 22 and 23, the milk from all quarters was examined with negative results. The hemolytic streptococcus had been replaced by the bovine non-hemolytic type. The milk was again examined on May 27 and 28, and the later findings confirmed. Little of significance could be obtained from the history of this cow except that it had been in the herd for 3 years. During the preceding lactation periods many attacks of mastitis had been noted. During the 2 months prior to our examination four attacks of mastitis of the right fore quarter had been reported. The hemolytic streptococcus was definitely of the human type as proved by the usual tests and, as it will be shown later, closely resembled those isolated from Cows 1452 and 1462. No record is available which indicates that this animal was responsible for an outbreak of scarlet fever.

DISCUSSION.

Heretofore contamination has been regarded as the usual means of spread of scarlet fever by milk. The findings in regard to septic sore throat, however, indicate that udder infection with the human streptococcus is far more likely. The same may be said of milk-borne epidemics of scarlet fever.

Unfortunately our examination was conducted after the epidemic had subsided. It is true though that when the milk from this farm was withheld from the general supply and the general supply pasteurized the outbreak subsided. When we made our examination the owner maintained that the milk from the left hind quarter of the infected cow was "all right" although he had withheld it from the general supply. It can be argued, however, that such milk was fully capable of causing severe illness among the consumers provided it entered the general supply. If 1 quart of milk drawn at the time when the streptococci were most numerous was mixed with the herd supply, the actual

dilution in this instance would amount to 1:100 since there were 12 cows contributing about 100 quarts. Our maximum count indicated well over 300 million per cc. in the spontaneous case and over 1 billion in the experimental inoculation. Assuming that the infective product was again diluted at least a hundred times at the distributor's the number of streptococci in even a small quantity of milk would be relatively high.

Spontaneous Case 1452 and experimental Case 1462 are examples of extremely severe infections and they indicate that the organism was highly virulent for the cow. The infection obliterated for practical purposes the primarily involved quarter. However the resulting general resistance in both instances was insufficient to protect other portions of the udder from infection. This argues for a prompt exclusion from the milking shed of cows with involved quarters since the organism may gain entrance to normal quadrants without exciting severe reactions.

In sharp contrast to these cases is that of Cow 4262 in which the organism was known to be present in the milk on two occasions 8 days apart. It probably inhabited the udder during this period. However it disappeared and was replaced by a bovine type and was not found subsequently. That this cow failed to cause trouble to consumers can be explained on several grounds, (1) that the milk contained relatively few organisms, (2) that the milk was mixed with that of a large number of cows (50 or 100) and sold in a large city where a few cases of scarlet fever would attract little attention, and (3) that when the milk became abnormal it was eliminated from the supply. This case argues for a prompt investigation of the milk of all cows when milk-borne infection is suspected, since a few days delay in instances of this kind may be sufficient for the disappearance of the streptococcus from the udder.

The period of incubation is of considerable practical importance. In regard to this period little is known in spontaneous infection. In all the experimental inoculations there was little to arouse suspicion during the first 24 hours although streptococci could be readily detected in the milk. After this time abnormalities in the appearance of the milk were apparent. It must be borne in mind that in these experiments the organisms were introduced into the milk cistern, and

although the number of streptococci administered was small, in all probability in the spontaneous disease the infective dose would be even smaller; and unless contaminated material was introduced by teat tubes or other means the organisms would probably be deposited at the meatus or about the ends of the teats. This indicates a somewhat longer incubation period than that observed in the experiments.

SUMMARY.

The clinical and bacteriological findings in two cows the udders of which became infected under natural conditions with hemolytic streptococci of the scarlet fever type are discussed. One of the cows was found in a herd supplying raw milk to a small town where a milk-borne outbreak of scarlet fever had occurred a short time before. When small numbers of the streptococcus obtained from this case were injected into the udder of a normal cow severe mastitis accompanied by a well marked general reaction resulted. Evidence leads to the conclusion that a severe attack of mastitis due to this organism in one quarter does not sufficiently immunize the other quarters to protect them completely since the streptococcus can be readily implanted in them. The secondary infections were much milder than the original process.

We wish to acknowledge our indebtedness to Mr. W. T. Eakins, Assistant Epidemiologist of the New Jersey State Department of Health, who furnished us with the history of the outbreak; also to Mr. J. V. Mulcahy who supplied us with cultures F. C. and M. B. obtained from the throats of milk handlers, and to Dr. I. H. Shaw for accompanying us to the farm and for other courtesies.

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UDDER INFECTION WITH STREPTOCOCCI OF THE SCARLET FEVER TYPE.

II. A STUDY OF THE SCARLET FEVER TYPE OF STREPTOCOCCI ISOLATED FROM THE UDDER OF THE COW.

By F. S. JONES, V.M.D., AND RALPH B. LITTLE, V.M.D.

(From the Department of Animal Pathology of The Rockefeller Institute for Medical Research, Princeton, N. J.)

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Our first paper (1) dealt with the clinical manifestations resulting from invasion of the udder with a hemolytic streptococcus of probable human origin. It was shown that the culture isolated from the udder was capable on inoculation into the udder of a second cow of producing severe mastitis.

It was also stated that the ability of the streptococcus to lysis blood in the test-tube, its pathogenicity for rabbits, and a limiting hydrogen ion concentration of pH 5.0 in dextrose broth differentiated it from the bovine streptococcus and indicated its human origin. The present paper deals with this phase of the question and a further correlation of these streptococci with those of the scarlet fever group.

Source of Cultures.

The cultures were drawn from several sources. Our own isolations from the udder of the spontaneous and experimental cases were regarded as the primary type. Those strains kindly furnished us by Mr. J. V. Mulcahy, Chief of the Bureau of Bacteriology of the New Jersey State Department of Health, who isolated them from the throats of milk handlers at the dairy, have been indicated as F.C. and M.B. Two cultures of scarlet fever streptococci of known origin were used, one (N.Y.V) furnished by Dr. A. R. Dochez, of the College of Physicians and Surgeons, Columbia University, and the other (Sc. 55) supplied by Dr. Anna F. Williams of the Department of Health of the City of New York. In addition three cultures of *Streptococcus epidemicus* are included in some of the comparisons. Of this type, C. 54 is the second bovine passage strain of the streptococcus isolated by Brown and Orcutt (2) during a milk-borne epidemic of septic sore throat. The others (744 and 4560) were obtained at various times by one of us (F.S.J.) from the udder of cows.

Morphological and Cultural Findings.

It can be said that little difference exists between the organisms isolated from Cows 1452, 1462, 4262, and the throats of F. C. and M. B. and the known scarlet fever strains. In all, chains of cocci up to 30 in number are not unusual. All are Gram-positive and all encapsulated. On the whole the capsules are considerably smaller than those of *S. epidemicus*. After 24 hours incubation in plate cultures containing 0.5 cc. of defibrinated horse blood and 10 cc. of agar prepared from veal infusion the surface colonies are round,

TABLE I.

The Fermentation Characters of the Streptococci Isolated from the Udder and from the Throats of the Milk Handlers.

Culture	Final hydrogen ion concentration in							Action on milk
	Dextrose	Lactose	Saccharose	Mannitol	Raffinose	Inulin	Salicin	
Cow 1452, L.H.Q.....	5.2	5.2	5.3	7.2	7.6	7.6	5.3	Firm coagulation.
“ 1452, R.H.Q. (before inoculation).....	5.2	5.2	5.2	7.2	7.6	7.6	5.4	“ “
Cow 4262, R.F.Q.....	5.0	5.0	5.0	7.4	7.4	7.6	5.3	“ “
“ 1462, L.H.Q.....	5.2	5.4	5.3	7.6	7.4	7.4	5.6	“ “
Throat F.C.....	5.2	5.2	5.0	7.2	7.6	7.6	5.3	“ “
“ M.B.....	5.2	5.3	5.3	7.4	7.4	7.6	5.4	“ “
Non-hemolytic mastitis streptococcus from Cow 1452, L.H.Q.....	4.5	4.6	4.6	7.0	7.6	7.6	4.7	“ “

flattened, translucent, and rarely exceed 1 or 1.5 mm. in diameter. They are surrounded by a sharp clear zone. The deep colonies vary in shape from round to biconvex and form the nucleus of beta hemolytic zones averaging 2.5 mm. in diameter. A further 24 hours incubation fails to increase the size of the colonies to any great extent although the hemolytic areas are increased to 3.0-3.5 mm. The colonies of *S. epidemicus* employed in the comparisons were larger, more sharply raised, and distinctly mucoid. The hemolytic areas were larger. The non-hemolytic streptococcus isolated from the udder of Cow 1452 is included in Table I for purposes of comparison.

To test the biochemic activities, all cultures were grown in fermented broth (pH 7.4), to which sufficient of the test substance had been added to make a 1 per cent solution, for 7 days. The results are recorded in Table I.

Table I indicates a close resemblance in the fermentation characters of the streptococci isolated from the spontaneous udder in-

TABLE II.
The Effect of the Cultures on Rabbits.

Rabbit No.	Weight	Intravenous injection—24 hr. broth culture	Result
	gm.		
1	1,010	1 cc. Cow 1452, L.H.Q., Culture A	Died. 28 hrs. Septicemia
2	2,050	1 " " 1452, " " B	" during night of 2nd day. Septicemia
3	1,105	1 " " 1452, R.H.Q., before inoculation	Died. 36 hrs. Septicemia
4	1,425	1 cc. Cow 1452, L.H.Q.	" on 4th day. "
5	1,575	1 " " 4262, R.F.Q.	Febrile reaction during first 7 days. Abscess right hock. Chloroformed on 10th day. Streptococci recovered from abscess
6	1,040	1 " culture from throat of F.C.	Febrile reaction during first 2 days. Bacteriemia during first 24 hrs. Chloroformed on 11th day. Abscess right hock. Vegetation on right auriculoventricular valve
7	1,130	1 " " " " M.B.	Febrile reaction throughout period Bacteriemia during first 3 days. Chloroformed on 11th day. Abscesses both elbows. 4 vegetations on right auriculoventricular valve

fections and those obtained from the throats of the handlers at the distributor's. All attacked dextrose, lactose, saccharose, salicin, and coagulated milk whereas the other substances were not fermented. It is of interest to note that the passage of strain Cow 1452 through the udder of Cow 1462 failed to change its fermentation characters. The acid production of the non-hemolytic bovine streptococcus offers a sharp quantitative comparison.

Mention has been made of the pathogenicity of our strains for rabbits. As soon as feasible after the isolations rabbits were injected intravenously with 1 cc. of a 24 hour broth culture. The results are recorded in Table II.

The cultures from Cows 1452 and 1462 were more virulent for rabbits than the others. That from Cow 4262 behaved about the same as did those isolated from the milk handlers. These streptococci tended to localize in the joints and heart valves.

It will be noted that the preceding characters indicate human origin, since the bovine streptococcus produces more acid and is not pathogenic for rabbits in similar doses. They afford no specific differentiation between *S. scarlatinæ* and *S. epidemicus*, although by inference it might be said that, since Culture 1452 was identical with those obtained from the throats of the milk handlers during an epidemic of scarlet fever, all three were of the scarlet fever type.

Further comparisons were regarded as essential. Attempts were made to group the strains by their agglutination affinities. Even though the cultures were grown for long periods at room temperature, as suggested by Shibley (3), and suspended in 0.2 per cent NaCl solution made alkaline with NaOH, the results were inconclusive.

The recent contribution of Lancefield (4) for the differentiation of streptococci by means of precipitin offered a means of separation. Lancefield has shown that immunization for long periods with broth cultures of streptococci results in the production of precipitin. For antigenic purposes in conducting the tests the growth from a liter of broth culture is suspended in salt solution to which sufficient N HCl is added to make a concentration of N/20. The suspensions are then extracted in a water bath at 100°C. for $\frac{1}{4}$ hour and neutralized with N NaOH. They are then freed of bacteria by centrifugation. When suitable quantities of such antigen are brought in contact with potent antiserum, the usual precipitin phenomena develop. When the fluids are mixed considerable precipitate is formed.

Rabbits were immunized by intraperitoneal injections of killed cultures followed by smaller doses of living organisms. For this purpose strains Cow 1452, F. C., Scarlet Fever N. Y. V, Scarlet Fever 55, and *S. epidemicus* Cow 4560 were used. Even after prolonged injection the sera of rabbits treated with strain Cow 1452 revealed only a weak precipitin. Scarlet Fever 55 failed to give rise to any

precipitin. A fair serum was obtained with N. Y. V and strong sera with F. C. and *S. epidemicus* 4560. In Table III we have recorded the reactions resulting from the addition of 0.15 cc. serum to 0.4 cc. antigen.

By means of Lancefield's procedure it was possible to divide the streptococci into two groups. Those associated with the milk-borne epidemic of scarlet fever—Cow 1452, Cow 1462, and F. C. and M. B.—

TABLE III.
Precipitation of Extracts of Streptococci with Various Antisera.

Streptococcic extract	Antisera			
	Rabbit immunized with			
	Culture Cow 1452	Throat culture F.C.	Scarlet fever streptococcus N.Y.V	<i>S. epidemicus</i> 4560
Cow 1452 L.H.Q.....	+	+++	+++	+-
" 1452 R.H.Q, before injection.....	+	+++	+++	+-
after injection.....	+	+++	++	+-
Cow 1462 L.H.Q.....	+	+++	++	+-
Throat F.C.....	+	+++	++	+-
" M.B.....	+	+++	++	+-
Cow 4262.....	+	++	++	+-
Scarlet fever 55.....	+	+++	++	+-
" " N.Y.V.....	+	+++	++	+-
<i>S. epidemicus</i> Cow 54.....	-	-	+-	+++
" " " 744.....	-	-	+-	+++
" " " 4560.....	-	-	+-	+++

* + indicates moderate contact reaction with definite flocculation after mixing; ++, a well defined contact with considerable flocculent precipitation after mixing; +++, the maximum reaction.

behaved like the scarlet fever streptococci N. Y. V and 55. The culture isolated from Cow 4262 had a similar antigenic affinity. The three strains of *S. epidemicus* comprised a separate group. There was a slight tendency to precipitation, as indicated by the +- reactions, when the extracts from the scarlet fever group were treated with *S. epidemicus* serum, but for purposes of classification these can be ignored since the reactions indicated by ++ and +++ were so marked as to be unmistakable.

In the course of the further correlation it proved possible in two instances by means of intracutaneous tests on persons susceptible to scarlet fever toxin to show that culture Cow 1452 produced a toxin similar to that of scarlet fever streptococcus N. Y. V and that this toxin was neutralized by scarlet fever antitoxin.

The following protocols are submitted.

Cultures 1452 and N.Y.V were grown for 5 days in flasks containing a shallow layer of broth. The cultures were centrifuged and the supernatant fluid passed through Berkefeld candles of V porosity. Dr. Richard E. Shope of this Department, who had never had scarlet fever, volunteered as a subject. One of the writers (F.S.J.) also proved susceptible to the scarlet fever toxin. The filtrates were diluted in salt solution and the equivalent of 1/100 cc. of filtrate injected intradermally. The same amount of each filtrate was mixed with 0.05 cc. of scarlet fever antitoxin and injected. In all there were four injections, (1) scarlet fever filtrate, (2) scarlet fever filtrate + scarlet fever antitoxin, (3) culture Cow 1452 filtrate, and (4) culture Cow 1452 filtrate + scarlet fever antitoxin.

Subject R.E.S. responded severely at Sites 1 and 3. Site 2 was negative and Site 4 revealed only a slight reddening without swelling, the redness subsiding rapidly.

Subject F.S.J. responded most severely at Site 3, and had a definite reaction at Site 1. Sites 2 and 4 revealed some reddening which rapidly faded.

DISCUSSION.

The evidence seems clear that a streptococcus indistinguishable from the scarlet fever type may, under certain conditions, gain access to the cow's udder and there produce characteristic inflammatory changes.

That the streptococci isolated from the cows under study fulfill the criteria outlined for human streptococci is shown by their ability to lysis blood in the test-tube, their final hydrogen ion concentration (5.0) in dextrose broth, and their pathogenicity for rabbits. Furthermore they may be differentiated from *S. epidemicus* by their antigenic complex when tested with specific precipitin. Extracts prepared in the manner described by Lancefield precipitated well with scarlet fever serum but failed to do so when mixed with *S. epidemicus* antiserum. The neutralization of the toxin of culture Cow 1452 by scarlet fever antitoxin is further evidence of similarity. As far as we could determine the passage through a second cow failed to markedly change the *S. scarlatinæ*-like character of the strains.

SUMMARY.

The streptococcus isolated from the udder of a cow on a farm where an outbreak of scarlet fever originated has been correlated with known scarlet fever strains. This streptococcus and another also isolated from the udder of a cow are indistinguishable in cultural characters and certain antigen affinities from *S. scarlatinæ*. Skin tests indicate that the strain isolated from the milk of the cow in a herd to which the scarlet fever epidemic was attributed produced a toxin which was neutralized with scarlet fever antitoxin.

We are indebted to Dr. Richard E. Shope of this Department for cooperation in making the skin tests.

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UDDER INFECTION WITH STREPTOCOCCI OF THE SCARLET FEVER TYPE.

III. THE INFLUENCE OF MILK ON THE GROWTH OF SCARLET FEVER STREPTOCOCCI.

By F. S. JONES, V.M.D.

*(From the Department of Animal Pathology of The Rockefeller Institute for
Medical Research, Princeton, N. J.)*

PLATE 38.

(Received for publication, March 9, 1928.)

The view usually held covering milk-borne epidemics of scarlet fever is that the streptococci originate in the throats of the milk handlers and through contamination gain access to the supply. It is difficult to explain on such grounds the heavy incidence of infection among the consumers of pooled supplies since relatively few organisms would gain access to limited amounts of milk, and when this was mixed with other milk the dilution would be so great that the probable incidence of human infection would be relatively small. It might be argued that the contaminating streptococcus would multiply rapidly in the milk provided the temperature was favorable and that the product reaching the consumer would thus contain large numbers of the streptococci. But to explain severe outbreaks of scarlet fever through milk contamination one would have to assume that the organism gained access in goodly numbers and multiplied rapidly. However certain experiments here to be reported indicate that streptococci of the scarlet fever type are acted upon adversely by milk.

During the observations in connection with the artificial inoculation of Cow 1462 with a streptococcus of the human scarlet fever type a peculiar phenomenon was observed. It was customary to plate the milk from the involved quarters after logarithmic dilutions in salt solution, the first plate of a series containing a 10^{-1} dilution, the second a 10^{-2} , until to the final plate milk diluted 10^{-6} was added.

Toward the end of the observation, when the numbers of streptococci per cc. were not excessive, it was noted that the first plate culture contained streptococci in smaller numbers than one would expect from the number found in the higher dilutions. Furthermore the colonies were very small with correspondingly small hemolytic zones; yet when such colonies were subcultured and replated colonies of the usual size with well defined hemolytic zones developed. Two explanations suggested themselves, first, that the colonies were too numerous in the plate, and second, that an inhibitory substance had developed in the quarter which was active in the 10^{-1} dilution but not in the 10^{-2} dilution. Further observations indicated that overcrowding was not responsible for the change in the character of the colonies. The inhibition appeared to be a property of the milk and as such worthy of more careful study.

EXPERIMENTAL.

The cultures employed have been described in the preceding papers. They were carried for stock purposes on agar slants to which a few drops of defibrinated horse blood had been added. For the experiments transfers were made into veal infusion broth and incubated 16 hours. Inasmuch as the rest of the procedure varied, some details of the individual experiments are given separately.

Experiment 1.—Milk from individual quarters of Cow 1462 was drawn directly into sterile bottles and after chilling freed of fat by centrifuging. That from the quarters artificially infected with the hemolytic streptococcus was combined and portions of this milk and of that from an uninfected quarter were heated at 60°C . for 20 minutes, while other portions were not heated. In addition the cow was bled on the day before the experiment and the serum collected.

The inhibitory activities of the milk and serum were then tested in the following manner. Serum or milk was added in amounts of 0.5, 0.25, and 0.1 cc. to Petri dishes containing 0.5 cc. of defibrinated horse blood. A loop of dilute broth culture of the streptococcus obtained from Cow 1462 was added to 10 or 12 cc. of melted agar cooled to 48°C . and the whole plated. The plates were incubated for 48 hours at 38°C .

Inasmuch as the unheated milk contained many native organisms giving rise to many colonies in the plates, the results were imperfect and for this reason they will not be considered in detail, although it was clear that even under such conditions the growth of the streptococcus was inhibited.

The results of the first experiment are given in Table I.

The fact seems established by the experiment that when milk from either the infected or normal quarters was added to plate cultures containing small numbers of hemolytic streptococci a distinct inhibition resulted. The colonies were smaller and their hemolytic activity greatly diminished or even entirely suppressed, as when 0.5 cc. of milk from the two involved quarters was incorporated in the culture. In the series in which blood serum was mixed and incubated

TABLE I.

The Influence of Blood Serum and Milk on the Size of Colonies and Hemolytic Activities of the Streptococcus from Cow 1462.

	Plate containing	
Blood serum	cc.	
	0.5	30 colonies varying from 1.5 to 1.8 mm. Hemolytic zones 4 mm.
	0.25	20 colonies average 1.8 mm. Hemolytic zones 4 mm.
Milk heated 60°C., from uninfected quarter (R. F.)	0.5	No growth
	0.25	6 colonies averaging 0.4 mm. Indistinct hemolytic zones 0.8 mm.
	0.1	5 colonies 0.4 to 0.6 mm. Hemolytic zones 1.25 to 1.75 mm.
Milk heated 60°C., from involved quarters, R. H. and L. H.	0.5	No growth in 24 hrs. 48 hrs., 5 tiny, non-hemolytic colonies
	0.25	30 colonies 0.25 mm. Hemolytic zones 0.6 mm.
	0.1	6 colonies 0.025 mm. Hemolytic zones 0.75 mm.

with the culture material there was no inhibition. One may infer that the inhibiting property was not derived from the blood.

It seemed possible that the organism employed might be unique in sensitiveness to the inhibitory action of milk. Furthermore the incorporation of as much as 0.5 cc. of milk to the blood agar plate might perhaps alter the nutritive character of the medium and thus prevent normal growth. The more elaborate procedure employed in Experiment 2 was devised to control these features.

TABLE II.

The Effect of Milk on the Character of the Growth of the Scarlet Fever Streptococcus.

Culture	Milk	Plate containing	
F. C.	R. F., filtered	cc.	
		0.5	No growth 24 hrs. 48 hrs., 10 colonies, tiny, surrounded by faint hemolysis
		0.25	384 colonies, average 0.4 mm. Hemolytic zones 0.75 mm., indistinct
	R. F., heated 58°C. 20 min.	0.1	345 colonies, average 0.6 mm. Hemolytic zones 1 to 1.25 mm., clear
		0.5	320 colonies less than 0.25 mm. Hemolytic zones barely perceptible
		0.25	384 colonies average 0.25 mm. Hemolytic zones 0.5 mm.
	R. H., filtered	0.1	350 colonies, average 0.6 mm. Hemolytic zones 1 mm.
		0.5	325 colonies barely visible $\times 9$. Non-hemolytic
		0.25	400 " " " $\times 9$. "
	R. H., boiled 5 min.	0.1	384 colonies, average 0.9 mm. Hemolytic zone 3 mm.
		0.5	428 colonies, average 0.9 mm. Hemolytic zone 3 mm.
		0.1	384 colonies, average 0.8 mm. Hemolytic zone 3 mm.
	None	0.5	
		0.25	
		0.1	
Scarlet Fever 55	R. F., filtered	0.5	90 colonies visible $\times 9$. Non-hemolytic
		0.25	576 colonies, less than 0.25 mm. Hemolytic zones 0.5 mm.
		0.1	580 colonies, average 0.5 mm. Hemolytic zones 2 mm.
	R. F., heated 58°C. 20 min.	0.5	No growth
		0.25	640 colonies less than 0.25 mm. Hazy hemolytic zones 0.5 mm.
		0.1	576 colonies 0.25 mm. Clear hemolytic zones 0.75 mm.
	R. H., filtered	0.5	No growth
		0.25	30 colonies visible $\times 9$. Non-hemolytic
		0.1	179 colonies 0.25 to 0.3 mm. Clear hemolytic zones 0.6 mm.
	Boiled 5 min.	0.5	428 colonies 0.8 to 1 mm. Clear hemolytic zones 3 mm.
		0.25	
		0.1	
	None	0.5	
		0.25	
		0.1	

Experiment 2.—Culture F. C. from the throat of a milk handler and Scarlet Fever Streptococcus 55 were employed in this experiment. Milk in separate bottles was obtained directly from the right fore and right hind quarters of Cow 1462. It was chilled and freed of fat. One portion of each was filtered through Berkefeld candle V, another lot heated at 58°C. for 20 minutes, and the remainder boiled for 5 minutes. The various lots in amounts of 0.5, 0.25, and 0.1 cc. were incorporated in the plate cultures. The effect on the growth of both strains is recorded in Table II.

The results of Experiment 2 resemble those of Experiment 1. 0.5 cc. of milk, filtered or heated at 58°C. for 20 minutes to rid it of native bacteria, proved sufficient to inhibit or entirely suppress the growth of the scarlet fever streptococcus. In certain instances although colonies of the organism developed in the Petri dishes no hemolysis occurred; nevertheless when such colonies were subcultured and replated in the blood agar mixture characteristic hemolytic colonies developed. The control to which 0.5 cc. of boiled milk was added failed to show appreciable inhibition.

Two series of the plate cultures were photographed. In order to afford a proper comparison they were magnified about 5 times. Figs. 1 to 5 are the photographs of the plate cultures of Strain F. C. without milk (Fig. 1), with boiled milk (Fig. 2), and the series in which 0.5, 0.25, and 0.1 cc. of filtered milk was added (Figs. 3, 4, 5). Figs. 6 to 10 show the effect of mixing milk heated at 58°C. for 20 minutes with the cultures of Scarlet Fever 55. Photographs of the two controls, one without milk and the other with boiled milk, are included for comparison.

It might be argued that the milk from Cow 1462 contained some immune property acquired as the result of partial recovery from infection. To test this possibility Experiment 3 was devised.

Experiment 3.—Milk was obtained directly from the udder of five normal cows chosen at random from those of a large herd. It was mixed and when freed of fat a portion was filtered, another heated at 58°C. for 20 minutes, and a third lot boiled for 5 minutes. It was then added in the usual amounts to the Petri dishes and its effect on cultures of Strain F. C. and Scarlet Fever V noted.

It was found after 48 hours incubation that the growth of Culture F. C. in the plates containing 0.5 cc. of either filtered milk or milk heated at 58°C. was completely inhibited. In plates containing 0.25 cc. either nearly complete inhibition occurred, or the colonies were too small to be seen with the unaided eye. Even

as little as 0.1 cc. of milk greatly diminished the size of the colony and the zone of hemolysis. The same could be said of Scarlet Fever V. In both series the addition of 0.5 cc. of boiled milk failed to diminish the number or size of the colonies although the hemolytic zones were a little smaller than in the control cultures which were made without milk.

It appears certain then that there is a natural inhibitory substance in milk which passes through a Berkefeld filter V and is not greatly injured when milk is heated at 58°C. for 20 minutes but inhibits the growth of streptococci of the scarlet fever type in plate cultures. The inhibition depends on the concentration of the milk but even when this is diluted as much as 1:100, as in the series in which 0.1 cc. of milk was mixed with 10 or 12 cc. of agar and 0.5 cc. of blood, its effect is readily visible.

Since cow's milk contains a principle which is inhibitory even when mixed with the culture medium, it seemed probable that exposure of the streptococci to the direct action of undiluted milk might result in definite destruction. To test this point milk was inoculated with the streptococcus and plated, in the manner outlined by Jones and Little (1), in the hope that the rate of growth or destruction could be measured. As might be expected from the preceding experiments, milk even when inoculated with 3,000 or 4,000 streptococci per cc., failed to show growth when plated in amounts of 1 cc. It was necessary to change the methods considerably before decisive experiments were obtained.

Experiment 4.—Milk from five cows chosen at random was mixed and freed of fat by centrifugation. A portion was heated at 58°C. for 20 minutes and the remainder boiled for 5 minutes. Both lots were then distributed into sterile agglutination tubes in amounts of 1 cc. The tubes were then separated into two groups each containing an equal number of tubes of the pasteurized and the boiled milk. Each tube of one group was inoculated with 1 loop of Strain F. C. diluted 500 times in broth. Those of the other group received a similar inoculation with Scarlet Fever V. Plate cultures were made by adding 0.25 cc. of milk to uniform amounts of blood and agar. Initial plates were poured and others after various intervals of incubation. The results of this experiment are recorded in Table III.

While there is some irregularity in the results recorded in Table III, the influence of the milk is evident. It is clear that fresh milk heated to 58°C. for 20 minutes actually prevents multiplication of

scarlet fever streptococci during incubation periods ranging from 2 to 48 hours. From the protocol it appears that the milk probably destroyed both strains of streptococci after an interval of 4 or 6 hours. To determine whether this was actually the case required further experimentation. It seemed possible that the organism subjected to the unfavorable influence of the milk heated at 58°C. was incapable of growth when plated because of the fact that the culture medium

TABLE III.

The Effect of Undiluted Milk on the Scarlet Fever Streptococcus.

Culture	Milk	Colonies developing in plate cultures containing 0.25 cc. milk						
		At once	After 2 hrs.	After 4 hrs.	After 6 hrs.	After 8 hrs.	After 24 hrs.	After 48 hrs.
F. C.	Heated 58°C.	No growth	243 Visible × 12	256 Visible × 12	No growth	No growth	No growth	No growth. Reaction pH 6.6
F. C.	Boiled	409	4,708 Whole plate he- molyzed	72,000	Innum- erable	Innum- erable	Innum- erable	Coagulates on boiling. pH 5.2
Scarlet Fever 55	Heated 58°C.	384	154 Visible × 12, non-he- molytic	No growth	No growth	No growth	No growth	No growth. pH 6.6
Scarlet Fever 55	Boiled	512	7,488	86,400	Innum- erable	Innum- erable	Innum- erable	Coagulates on boiling. pH 5.4

contained 0.25 cc. of the inhibiting milk. This factor, added to the effect of previous exposure, might still be insufficient to kill the organism although suppressing its multiplication. That the same milk when boiled for 5 minutes was well adapted as a culture medium is obvious in the protocol, since multiplication was noted throughout the series.

In the next experiment only a trace of milk was added to the plate cultures.

Experiment 5.—Milk was obtained from the same cows as in Experiment 4. It was handled in a similar manner. After distribution in agglutination tubes in amounts of 1 cc., each tube was inoculated with a loop of broth culture diluted 500 times. Each tube before plating was centrifuged at high speed for 15 min-

TABLE IV.
Streptococci Surviving after Incubation in Milk.

Culture	Milk	Colonies developing in plate cultures						
		At once	After 2 hrs.	After 4 hrs.	After 6 hrs.	After 8 hrs.	After 24 hrs.	After 48 hrs.
F. C.	Heated 58°C. 20 min.	205	5	11	11	Sterile	Sterile pH 6.6	Sterile pH 6.6
F. C.	Boiled 5 min.	218	8,960	86,400	Innum- erable	Innum- erable	Innumerable pH 6.0 Thickens on boiling	pH 5.4 Coagulates on boiling
Scarlet Fever V	Heated 58°C. 20 min.	217	205	192	205	77	11 pH 6.6	Sterile pH 6.6
Scarlet Fever V	Boiled 5 min.	218	4,992	46,080	Innum- erable	Innum- erable	Innumerable pH 6.0	pH 5.6 Coagulates on boiling

TABLE V.
The Effect of Milk on Scarlet Fever Streptococci at 3° and 4°C.

	Before refrigeration	After refrigeration	
		24 hrs.	48 hrs.
Culture F. C. in milk heated 58°C. 20 min.....	205	24	12
“ “ “ boiled milk.....	218	300	412
“ Scarlet Fever V in milk heated 58°C. 20 min.....	217	156	102
“ “ “ “ boiled milk.....	218	130	166

utes. The bulk of the milk was drawn off and 1 cc. sterile salt solution added and thoroughly mixed. The mixture was then withdrawn and added to the Petri dishes. In this way it was hoped that so little milk would be added to the medium that its effect would be negligible. The majority of the tubes were incubated

and plates prepared at indicated intervals. Some of the tubes were refrigerated at 3-4°C. and their contents plated by the same method after 24 and 48 hour intervals. The colonies were counted after an incubation of 48 hours in the blood agar medium. The results are given in Tables IV and V.

It is evident that the scarlet fever streptococcus fails to multiply in mixed milk provided the milk has not been heated sufficiently to destroy the inhibitory substance. It is also true that the principle in milk actually destroys the organism. The lethal effect is most marked at 38°C. At this temperature Culture F. C. was killed after 8 hours incubation. Scarlet Fever Streptococcus V was more resistant to the action of milk, since there was a more gradual diminution in the number of organisms, but after 48 hours none survived.

When the same cultures were exposed to the action of milk in the refrigerator Strain F. C. again proved more susceptible since only about 10 per cent of the streptococci survived for 24 hours, whereas culture Scarlet Fever V was not appreciably affected by the milk during a refrigeration of 24 hours or even 48 hours.

When the experiment was repeated at the temperature of the room, the number of streptococci was definitely diminished although not so greatly as at incubator temperature. The surviving streptococci failed to approach in number those implanted in milk and refrigerated.

DISCUSSION.

It can be said that milk heated at 58°C. for 20 minutes or filtered through the coarsest Berkefeld filter possesses the property of inhibiting the growth of the scarlet fever streptococcus. It also is true that the principle is sufficiently active to destroy certain of the organisms. That this phenomenon cannot be attributed to the lack of adaptation of the organism for growth in the food mixture represented by milk is amply shown by the behavior when boiled milk is used. It must be recognized that this activity is not specific for the streptococcus since it has been shown by others that other types of organisms are inhibited. However, it appears to be particularly potent for streptococci especially those of the scarlet fever type. The inhibitory and lethal effect of milk on the streptococcus is most marked at temperatures approaching that of the body. When artificially infected milk is stored at 3° or 4°C. more of the streptococci survive.

Since the evidence points to the udder as the source of origin of the inhibitory principle it is not surprising that the action of the latter should be most effective at a temperature about that of the organ in which it originates.

Milk added to plate cultures entirely prevents the development of colonies or so changes the appearance of the colonies as to make them unrecognizable. In many instances signs of hemolysis are not to be seen. Even when as little as 0.1 cc. of milk is added to the medium the surrounding hemolytic zone is so small that the colonies can easily be mistaken for the bovine type. These facts must be borne in mind when mixed milk is examined for human hemolytic streptococci.

Since the experimental evidence indicates strongly that milk, provided it is not heated at too high a temperature, will inhibit the growth or kill the scarlet fever streptococcus, the opinion that severe outbreaks of scarlet fever result from human contamination of milk must be viewed with considerable doubt. It might be objected that the experiments were artificial. But they were optimum conditions for the growth of the streptococcus and other organisms which would rapidly sour the milk under natural conditions were excluded. The change from a broth medium to one containing milk would appear to be no greater than that from a human throat to raw milk. That a few individuals may contract the disease through direct human contamination of milk is possible, but the occurrence of epidemics would imply a heavy inoculation of the milk. Infection of the udder of a single cow with the scarlet fever streptococcus and the resultant shedding of large numbers of the organisms into the milk,—phenomena recorded in our foregoing papers,—afford a more reasonable explanation of milk-borne epidemics.

SUMMARY.

The experiments indicate that milk filtered through a Berkefeld candle V or heated at 58°C. for 20 minutes when added to blood agar plate cultures interferes with the development of colonies of the scarlet fever streptococcus. The observed inhibition is proportional to the amount of milk. When the approximate milk dilution in the Petri dish is 1:20 or 1:25 growth of the organisms is completely suppressed or only a small proportion of non-hemolytic colonies develop.

As the amount of milk is decreased the colonies become larger and their hemolytic zones more pronounced, although even when the final dilution of milk reaches 1:100 or 1:125 only colonies easily mistaken for the narrow zoned bovine streptococci appear. The effects upon the surviving organisms would appear to be transient since both the non-hemolytic colonies and those with small zones manifest the original hemolytic properties when transferred to other media. When scarlet fever streptococci are added in small quantities to milk heated at 58°C. for 20 minutes and incubated growth is inhibited. If the period is prolonged the streptococci are killed. On refrigeration of such mixtures some of the streptococci are killed but others survive the test period.

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EXPLANATION OF PLATE 38.

Magnification \times about 5.

FIG. 1. Culture F. C. after 48 hours incubation in blood agar plate culture.

FIG. 2. Culture F. C. after 48 hours incubation in blood agar plate culture + 0.5 cc. boiled milk.

FIG. 3. Culture F. C. after 48 hours incubation in blood agar plate culture + 0.5 cc. filtered milk. There are no colonies of sufficient size to be detected at the magnification given.

FIG. 4. Culture F. C. after 48 hours incubation in blood agar plate culture + 0.25 cc. filtered milk. Note the difference in size of colonies in this plate when compared with those in Figs. 1 and 2.

FIG. 5. Culture F. C. after 48 hours incubation in blood agar plate culture + 0.1 cc. filtered milk. The colonies and hemolytic zones are larger than those in Fig. 4 but not as large as those in Figs. 1 and 2.

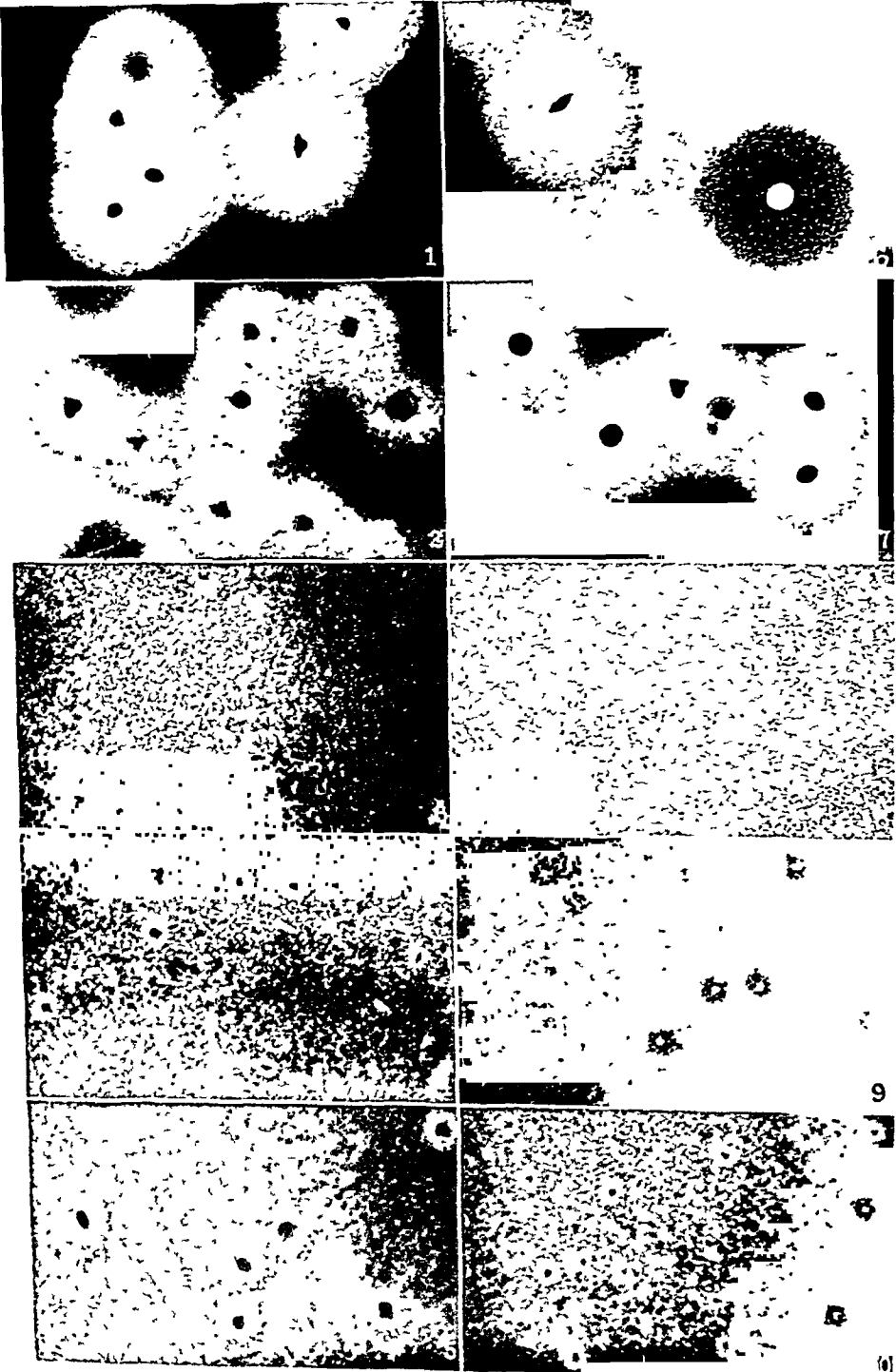
FIG. 6. Culture Scarlet Fever 55 after 48 hours incubation in blood agar plate culture.

FIG. 7. Culture Scarlet Fever 55 after 48 hours incubation in blood agar plate culture + 0.5 cc. boiled milk.

FIG. 8. Culture Scarlet Fever 55 after 48 hours incubation in blood agar plate culture + 0.5 cc. milk heated at 58°C. for 20 minutes. No growth visible at this magnification.

FIG. 9. Culture Scarlet Fever 55 after 48 hours incubation in blood agar plate culture + 0.25 cc. milk heated at 58°C. for 20 minutes. Note the small size of colonies and character of hemolytic zone as compared with Figs. 6 and 7.

FIG. 10. Culture Scarlet Fever 55 after 48 hours incubation in blood agar plate culture + 0.1 cc. milk heated at 58°C. for 20 minutes. The colonies and hemolytic zones are larger than those in Fig. 9 but not as large as those in Figs. 6 and 7.



Photomicrographs of cells.

(Gross: Letter infection with streptococci. III.)

THE REGENERATION OF AUTOPLASTIC LYMPH NODE TRANSPLANTS.

BY HENRY L. JAFFE, M.D., AND MAURICE N. RICHTER, M.D.

(From the Laboratory Division of the Hospital for Joint Diseases and the Pathological Laboratory of Bellevue Hospital, New York.)

PLATES 39 TO 41.

(Received for publication, March 22, 1928.)

This work was undertaken as a companion study to that on transplantation of thymus previously reported (1). In spite of the extensive literature on tissue transplantation we found no references to the transplantation of lymph nodes. Since the results of these experiments were to be compared with those of thymus transplants in guinea pigs, we carried out the first experiment on this species. Our experiments were unsuccessful because the transplantation of cervical lymph nodes of stock guinea pigs invariably led to abscess formation, which, we believe, was due to the use of lymph nodes already infected. The necrotic tissue which results from transplantation is a favorable medium for the growth of bacteria present in the nodes. Even the use of young guinea pigs, about 25 days of age, frequently gave the same result. We abandoned the experiments on guinea pigs, and used albino rats, which are quite resistant to pyogenic infection. We used rats between 3 and 5 weeks of age, and transplanted cervical nodes which were normal in gross appearance. Successful transplants were obtained in over 90 per cent of the cases. Whole lymph nodes were planted in pockets of the abdominal wall, by the same technique that was used in the transplantation of thymus.

In all, 31 albino rats were used, in each of which two nodes were transplanted simultaneously. In this series of 62 transplantations there were but four instances of infection.

The transplants were studied at about 24 hour intervals from the 1st to the 12th days, and at longer intervals up to 35 days. Most of the plants were removed between the 2nd and 7th days, as this is the period of most active regeneration.

The tissues were fixed in Bouin's fluid, Helly's fluid, or alcohol. The Bouin- and some of the Helly-fixed tissues were embedded in paraffin and stained with hematoxylin and eosin, Van Gieson, iron-hematoxylin, or phosphotungstic acid-hematoxylin. The plants fixed in alcohol were stained with pyronine and methyl green. Some of the Helly-fixed nodes were sectioned in celloidin, and stained with azure B-methylene blue-eosin (2). This last method gave the best preparations for studying the details of regeneration.

OBSERVATIONS.

Minor differences were observed in transplants of the same age even in the same animal. The following account is an approximation of the changes observed with reference to the time after transplantation.

At the end of 24 hours, there is extensive necrosis in the center of the transplant involving mainly the lymphocytes. At the periphery of the node some normal appearing lymphocytes are seen. Surrounding the node is a space, probably the peripheral lymph sinus of the transplanted node, which in some areas contains a few lymphocytes. Most of the reticulum of the transplant is preserved, though obscured in the central portions by the cellular debris. The vessels in the tissue around the transplant are not dilated, and the only reaction to the transplant is a slight polymorphonuclear infiltration between the muscle fibers about the transplant.

By the end of the 2nd day, three well defined areas are visible in the transplant: a central necrotic area, a peripheral margin of well preserved tissue, and an intermediate zone of hyperplastic reticulum (Figs. 1 and 2). Considerable liquefaction and absorption have taken place, so that the necrotic area is reduced in size. The reticulum contains numerous mitotic figures. The marginal zone contains mainly small lymphocytes and an occasional large lymphocyte. Mitotic figures in the lymphocytes are difficult to find. At the hilus of the node dilated vessels filled with blood penetrate the transplant, and their subdivisions extend to the periphery. The cellular reaction about the transplant is not extensive.

During the 3rd day, the necrotic mass becomes smaller, and the reticulum becomes more prominent. Large phagocytic cells in the reticulum aid in the removal of the debris. At the margin is a zone of small lymphocytes, among which are a few large lymphoid cells. Large lymphoid cells are formed from the reticulum in areas where reticulum cell hyperplasia is most prominent, and are therefore formed extravascularly. The formation of lymphocytes from tissue cells is also observed in the connective tissue outside the transplanted node.

During the 4th day, the debris is almost completely removed. The transplant now appears more like a normal lymph node, with very vascular connective tissue at the hilus (Fig. 3). Extravascular formation of lymphocytes from tissue cells is observed in areas both in and outside of the node. The peripheral lymphocytic

zone is wider. There are no germinal centers, but scattered small groups of large lymphoid cells, identical with germinal center cells, are observed. There is a tendency for the lymphocytes to be arranged in cords, extending from the periphery to the medullary portion.

After 5 days, regeneration is nearly complete. The transplant now appears as a normal lymph node. The lymph capillaries at the hilus are filled with lymphocytes and are efferent vessels (Fig. 4). The reticulum is still prominent, and mitoses may be observed. Large lymphocytes are scattered among the smaller cells of both the cortex and medulla.

At 6 days the node is completely regenerated. The efferent capillary lymph vessels previously observed may be traced from the hilus into the surrounding tissue.

At periods from 6 to 35 days, sections show that the transplant persists as a fully developed lymph node in the muscles of the abdominal wall. No retrograde changes were noted (Figs. 5 and 6).

DISCUSSION AND SUMMARY.

The reticulum plays an important part in the regeneration of lymph nodes autoplastically transplanted into the abdominal wall of the albino rat. The necrosis which follows transplantation involves mainly the lymphocytes. A margin of lymphocytes is preserved only at the periphery. This may be due to early lymphatic connection with the marginal sinus of the node. The reticulum cells are apparently more resistant since for the most part they do not degenerate. There are three possible origins of the lymphocytes of the regenerated node. They may arise by proliferation of lymphocytes retained at the periphery of the plant. The presence of a few mitotic figures among lymphocytes in the marginal region confirms the possibility of this mode of origin. They may also be derived from lymphocytes brought into the transplant by the blood or lymph circulation. Though some lymphocytes are present in the marginal sinus, and an occasional lymphocyte is seen in the capillaries which enter at the hilus, we believe that this source of origin of the lymphocytes is negligible. On the other hand, the hyperplastic reticulum appears to be the important source of lymphocyte production. These may be derived from the reticulum directly as small lymphocytes, or may be formed through the intermediary stage of large lymphoid cells. This capacity is not limited to lymphatic reticulum, as small foci of lymphocyte formation are found in the connective tissue in the vicinity

of the transplants. The reticular origin of lymphocytes is most easily observed in the earlier stages of regeneration before the picture is obscured by the numerous small lymphocytes.

Two structures in the regenerating node are directly traceable to the same structures of the transplant. These are the marginal sinus and the hilus. The marginal sinus of the transplant is preserved, and is probably an important means by which lymphatic communication is established with the surrounding tissue. The hilus is the site of entry of the blood vessels, and the hilus of the regenerated node is the same as that of the transplant.

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EXPLANATION OF PLATES.

PLATE 39.

FIG. 1. 2 days. Shows three well defined areas. Central necrotic zone, zone of well preserved lymphocytes, and intermediate zone of hyperplastic reticulum. Hematoxylin and eosin. $\times 37$.

FIG. 2. Higher magnification of the three zones shown in Fig. 1. Hematoxylin and eosin. $\times 112$.

PLATE 40.

FIG. 3. 3 days, 17 hours. The cellular debris has practically disappeared and its place is taken by a vascular reticulum with many newly formed capillaries. There is a peripheral zone of normal appearing lymphocytes. Eosin-azure B-methylene blue. $\times 72$.

FIG. 4. 5 days. Almost complete regeneration, but with prominent reticulum in the center. Efferent lymph channels contain many lymphocytes. Hematoxylin and eosin. $\times 55$.

PLATE 41.

FIG. 5. 7 days. Complete regeneration of transplant. Hematoxylin and eosin. $\times 32$.

FIG. 6. 21 days. Regenerated lymph node transplant after 21 days. No retrograde changes. Hematoxylin and eosin. $\times 37$.



FIG. 1.



FIG. 2.



FIG. 3.



FIG. 4.

Osaka and Fisher. Autoplastic lymph node transplants.

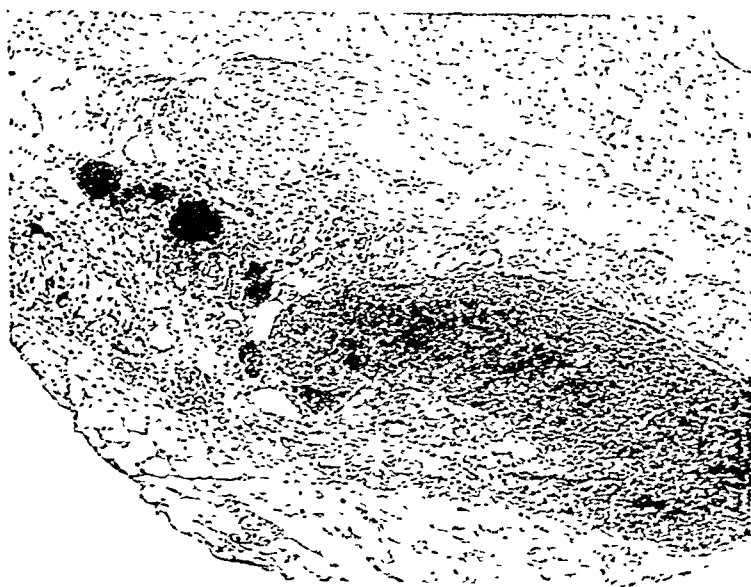


FIG. 5.

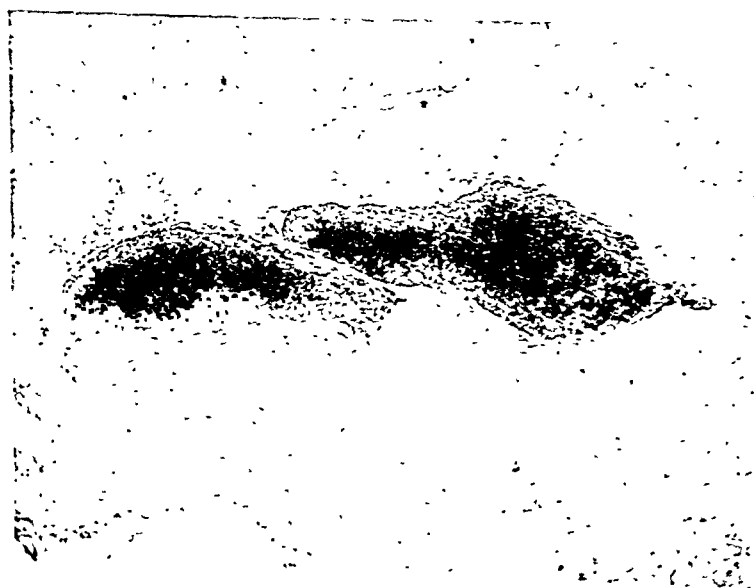


FIG. 6.

Udell and Richter. Autoplastic lymph node transplants.)

THE AUTOPLASTIC TRANSPLANTATION OF TISSUES INTO THE BONE MARROW CAVITY.

I. THE THYMUS.

By MAURICE N. RICHTER, M.D., AND HENRY L. JAFFE, M.D.

*(From the Pathological Laboratory of Bellevue Hospital and the Laboratory Division
of the Hospital for Joint Diseases, New York.)*

PLATES 42 TO 44.

(Received for publication, March 22, 1928.)

The relation of lymphoid tissue to bone marrow is not definitely known. According to the adherents of the dualistic theory of blood formation these two tissues are independent or even antagonistic to each other. Thus the possibility of a lymphocyte differentiating into a myeloid cell is denied.

The unitarian theory of blood cell development postulates a close relationship between the lymphoid and myeloid tissues, and lymphocytes are considered capable of differentiating, directly or indirectly, into myeloid cells. Maximow (1), Downey (2), and others have reported such findings. Downey has recently published a comprehensive review on this subject. Maximow (3) induced this change experimentally by the addition of cell-free bone marrow extracts to lymphoid tissue cultures.

We transplanted tissues into the bone marrow cavity, in direct contact with actively growing marrow, to study the effects of the marrow on the transplanted tissues, and of the tissues on the bone marrow. Three tissues of lymphoid nature—lymph node, spleen, and thymus—were transplanted. It was thought that if these tissues were genetically and biologically different, different reactions might be produced both in them and in the bone marrow on transplantation.

The lymph nodes are composed almost entirely of lymphocytes and their supporting stroma. Cytologically the spleen differs from lymph nodes, in that a small amount of myeloid tissue may be present, though overshadowed by the lymphocytes. The lymphoid nature of the

thymus is questionable because opinion is not unanimous concerning the "lymphoid" nature of the cortical cells. By the introduction of these tissues into the marrow cavity similarities and differences in their reactions were observed. This paper deals with transplantation of the thymus.

Maximow (4), Danchakoff (5), Hammar (6), and others regard the small thymic cells as lymphocytes. Stöhr (7), Jaffe (8), and others regard them as epithelial elements. In experiments on myeloid metaplasia Danchakoff reported the transformation of thymic cortical cells into granular myelocytes, a change supposedly characteristic of lymphocytes and their precursors.

The literature on transplantation of organs into the bone marrow is scant, and we have found no references to transplants of thymus, lymph nodes, or spleen in this situation.

Methods.

Young guinea pigs 8 to 11 weeks of age were used. These animals were reared in the laboratory under hygienic conditions, and were apparently healthy. Under ether anesthesia with aseptic precautions both lobes of the thymus were removed, and placed immediately in sterile physiological salt solution kept at about 38°C. An osteotomy was then performed on the upper third of each tibia. The marrow cavity of the upper third of the bone was curetted and wiped out with gauze. The thymus was then introduced into the cavity. Thirteen animals, in which the wounds healed by primary union, were used for this study. In six animals several fragments of thymus not more than 2 mm. in diameter were introduced into each marrow cavity, and in seven cases one large piece of thymus sufficient to fill the entire defect was inserted. The bone flap was replaced whenever possible, and the wounds were closed with fine silk.

The animals were killed at intervals of from 1 to 10 weeks after operation. The tibia was sawed through with a fine jeweler's saw at several levels, fixed in Helly's fluid, decalcified in 5 per cent nitric acid, sectioned in paraffin, and stained with hematoxylin and eosin.

The results of the transplantation of several small pieces were essentially the same as those which followed the transplantation of one large piece. Therefore both series of experiments are described together.

OBSERVATIONS.

At the end of 1 week, the transplanted thymus occupied the center of the marrow cavity and showed extensive degeneration of the small thymic cells and the Hassall's corpuscles, the thymic reticulum remaining for the most part intact. At several places in the peripheral portion of the transplant the thymic reticulum was hyperplastic and contained mitotic figures. A layer of loose cellular and vascular granulation tissue, in which new bone was forming, separated the thymus from the bone cortex. (Fig. 1.)

By the 2nd week much of the debris had been cleared away mainly by the process of liquefaction. Phagocytes were present in remarkably small numbers. At this time the outstanding feature in the transplant was the proliferating reticulum, with some round cells and an occasional polymorphonuclear leucocyte scattered in its meshes. During the 2nd week the small thymic cells reappeared and increased in number. The reticulum formed cell masses, the centers of which degenerated resulting in typical Hassall's corpuscles. We found no evidence of Hassall body formation from capillary endothelium as recently described by Jordan (9). In this stage new bone trabeculae were forming around the transplant. (Fig. 2.)

During the 3rd week the thymus was usually completely regenerated, forming large cellular lobules, some of which showed differentiation into cortex and medulla. The thymic lobules were richly cellular and the small round thymic cells predominated. The Hassall's corpuscles were well developed. The regeneration of the thymus was complete in that all the structures of the normal thymus in their normal morphological relationship were reproduced. The regeneration, however, fell short of reproducing as active and cellular a thymus as was introduced. This is in marked contrast to the results obtained when thymus is autoplastically transplanted into the abdominal wall (8). The bony trabeculae observed in the previous stage formed an incomplete bony capsule around the thymus. Where the bony wall was lacking, a thin layer of fibrous tissue separated the thymus from the marrow. (Figs. 3 and 4.)

By 6 weeks the bony capsule was fully developed and completely enclosed the thymus. The development of this bony capsule not

only prevented further growth of the thymus, but led to its atrophy. As the gland atrophied there was replacement fibrosis and Hassall's corpuscles disappeared. (Fig. 5.)

By 8½ weeks only a tiny fragment of thymus remained which was separated from the marrow cavity by a capsule of newly formed bone (Fig. 6). The thymus was markedly atrophic, the cortical cells had almost completely disappeared, and practically nothing remained but a stroma consisting mainly of connective tissue, in the center of which was a small group of round cells. Reticulum cells, if present, could not be differentiated from the cells of the fibroblastic stroma. No Hassall's corpuscles were seen. A few foreign body giant cells were present in the stroma.

In sections made at the end of 10 weeks, no thymic tissue was found.

SUMMARY.

When the thymus is autoplastically transplanted into the marrow cavity of the tibia of a guinea pig, the transplant degenerates, regenerates, and finally atrophies. The degeneration and regeneration of the thymus when so transplanted are similar to the homologous changes occurring when it is autoplastically transplanted into the abdominal wall. For comparative study the material used in previously published work on transplantation into the abdominal wall was reviewed. A difference in the final result of transplantation of thymus in these two situations is observed. In the abdominal wall the thymus regenerates and persists apparently indefinitely. Abdominal wall thymus transplants more than a year old have been observed. In the bone marrow cavity, the regeneration, which is slower and less abundant, is followed by an atrophy of the gland, and finally its complete disappearance. This atrophy is, we believe, the result of the encasement of the thymus in a bony capsule. That the genetic character of the transplanted tissue has much to do with the success of the transplantation in the marrow, is evident from the fact that some other tissues regenerate without encapsulation, even though they may finally disappear.

The blood clot about the thymus which fills the rest of the curetted cavity, is quickly vascularized and replaced by regenerated cellular bone marrow and newly formed bony trabeculae.

The difference in the fate of the thymus when transplanted in the abdominal wall and in the marrow, is probably due to differences in the reciprocal relations of the thymus to the tissues in those situations. From experience gained in the transplantation of other tissues into the marrow cavity, we have learned that encapsulation of the transplant does not occur with all tissues. The reaction of the marrow to transplanted thymus is similar to its reaction to any tissue foreign to it.

CONCLUSIONS.

1. The thymus when transplanted autoplastically into the bone marrow undergoes complete but not abundant regeneration.
2. The histological structure of the regenerated thymus is the same as that of the normal thymus despite its situation in the marrow cavity.
3. We have noted no metaplasia of the thymic round cells into myelocytes.
4. The regeneration of the bone marrow in the curetted cavity is not influenced by the presence of the thymus.
5. The regeneration of the thymus is accompanied by its enclosure in a fibrous and bony capsule which prevents extensive development and causes pressure atrophy of the transplant.

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8. Gottesman, J. M., and Jaffe, H. L., Studies on the histogenesis of autoplasic thymus transplantations, *J. Exp. Med.*, 1926, xliii, 403.
Jaffe, H. L., Autoplasic thymus transplants. II. With particular reference to the regeneration of the reticulum cells and the formation of Hassall's corpuscles, *J. Exp. Med.*, 1926, xliv, 523.
9. Jordan, H. E., and Horsley, G. W., The significance of the concentric corpuscles of Hassall, *Anat. Rec.*, 1927, xxxv, 279.

EXPLANATION OF PLATES.

PLATE 42.

FIG. 1. 7 days. Cross-section of the tibia at the level of the transplant showing several lobules of degenerated thymus. The section also shows the replaced bone flap. Hematoxylin and eosin. $\times 18$.

FIG. 2. 14 days. Regenerating thymus is surrounded by newly formed endosteal trabeculae. Hematoxylin and eosin. $\times 18$.

PLATE 43.

FIG. 3. 24 days. The regeneration of the thymus has reached its maximum. Hassall's corpuscles and medullary centers are seen. Marrow has regenerated between the trabeculae, and the bony encapsulation is progressing. Hematoxylin and eosin. $\times 44$.

FIG. 4. Higher magnification of the regenerated thymus shown in Fig. 3. Typical Hassall's corpuscles are seen. Hematoxylin and eosin. $\times 100$.

PLATE 44.

FIG. 5. 41 days. The encapsulation of the thymus has excluded it from the marrow cavity. The organ lies between the osteotomy flap and the newly formed endosteal trabeculae which separate it from the regenerated marrow. Retrograde atrophy of the thymus has set in. Hematoxylin and eosin. $\times 22$.

FIG. 6. 63 days. The thymus is completely surrounded by a bony capsule connected with the cortex. There is almost complete atrophy of the thymus. Hematoxylin and eosin. $\times 25$.



FIG. 1.

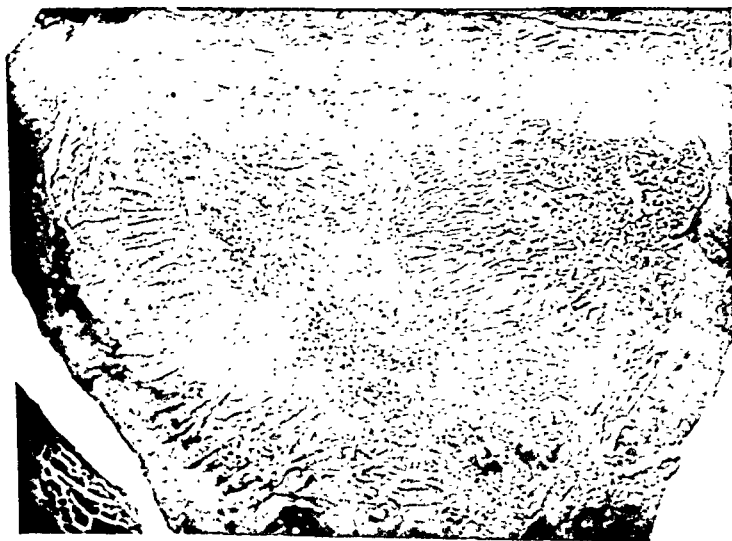


FIG. 2.

(Richter and Jaffe: Transplantation into bone marrow cavity. 1.)

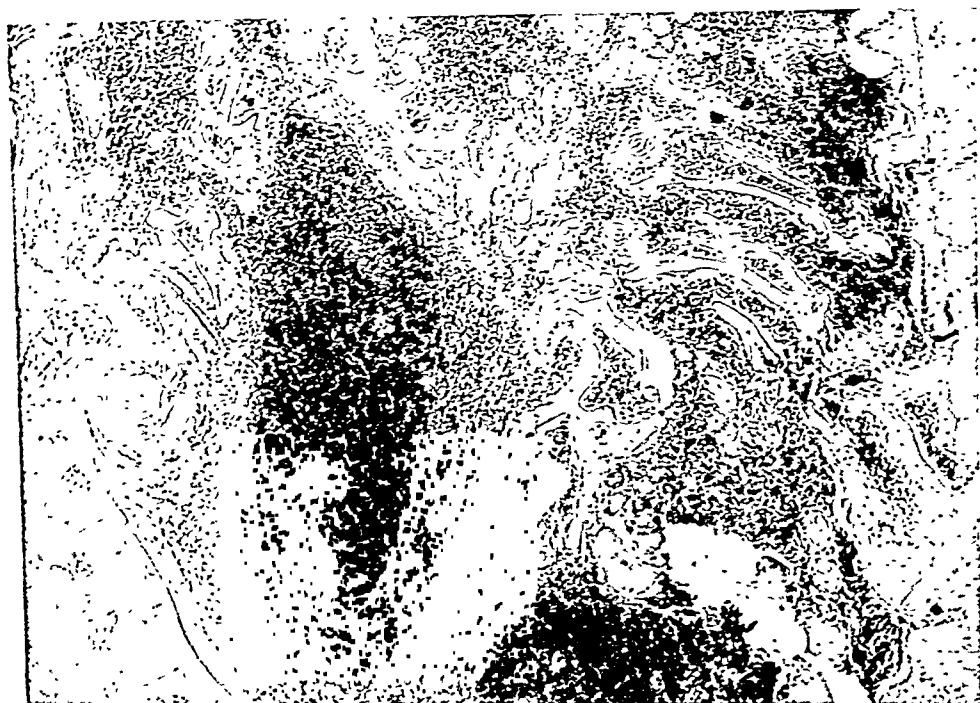


FIG. 3.



FIG. 4.

(Richter and Jaffe) Transplantation into bone marrow cavity. 13



FIG. 5.

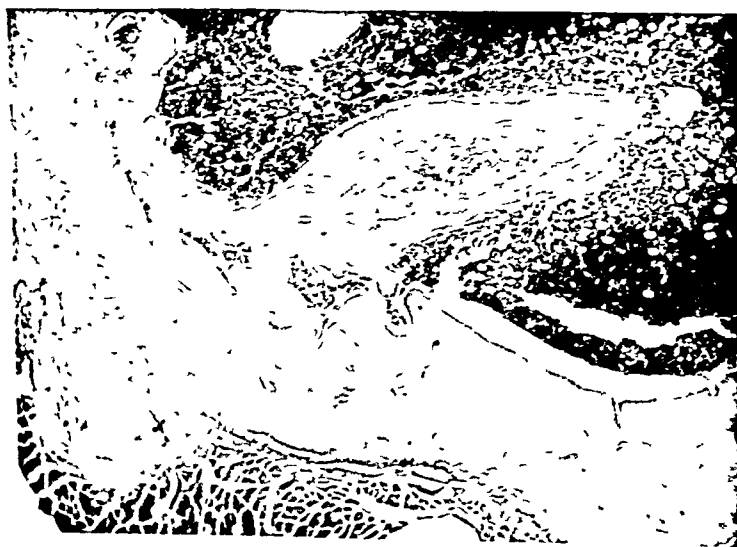


FIG. 6.

(Halter and Jaffe. Transplantation into bone marrow cavity. 1)

SURFACE TENSION OF SERUM OF THE SENSITIZED GUINEA PIG.

I. SURFACE TENSION CHANGES INCIDENT TO THE PROCESS OF SENSITIZATION.

By SUSAN GRIFFITH RAMSDELL.

(From the Research Institute of Cutaneous Medicine, Philadelphia.)

(Received for publication, March 5, 1928.)

In a study of surface tension in the immunized rabbit, du Noüy¹ found a certain definite progressive change to take place, but concluded that this change is "not directly correlated to the presence of antibodies, but that the two phenomena are simply co-existent at the beginning of immunization."²

This conclusion suggested a study of surface tension changes in the serum of the guinea pig incident to the process of sensitization, since the early stage in immunization in the rabbit—when tissue changes take place and when the protective mechanism of circulating antibodies is not yet in operation—is comparable to the stage generally accepted as prevailing as a permanent state in the guinea pig. Zunz and la Barre,³ using the drop method of measurement, did not observe any change in surface values as a result of serum sensitization. But it is to be noted that an examination was not made until after a period of 21 days following the sensitizing dose.

¹ du Noüy, P. L., *J. Exp. Med.*, 1925, xli, 779.

² du Noüy, P. L., Surface equilibria of biological and organic colloids, Chemical Catalog Company, New York, 1926, 120.

This volume presents the collected studies of du Noüy to which reference is made in this paper. The original papers appeared in the *J. Exp. Med.*, 1922, xxxv, 575, 707; xxxvi, 115, 547; 1923, xxxviii, 87, 659; 1924, xl, 129; 1925, xli, 663, 779; xlii, 9.

³ Zunz, E., and la Barre, J., *Compt. rend. Soc. biol.*, 1925, xcii, 223.

Technic.—The apparatus and technic as described by du Noüy were generally employed, with the following variations:⁴

1. An extension of the table carrying the watch-glass was made by superimposing a revolving table of wood large enough to carry five glasses.

2. Distilled water with a surface tension measurement of 73.0 dynes / cm. or more was accepted as meeting the requirements of a technic where only comparable values were of importance.

3. Careful checking of saline and glassware was carried out at the beginning of each test by reading a sample of saline which had been passed through all glassware. In addition, a sample of saline was read in each watch-glass.

4. Samples of sera which did not give an initial reading within 1 dyne of that of water were discarded.

5. In the second part of the study, dilutions of whole blood were used instead of serum or plasma. Only comparative results were required in the study, and whatever error the presence of blood cells might occasion was considered a constant factor.

EXPERIMENTS.

The work of du Noüy was repeated with three rabbits in the process of anti-horse serum precipitin production and with two untreated rabbits. He found that the static surface tension value of the serum of the treated rabbits was appreciably lowered. (This difference in the initial and the 2 hour, or static, readings he refers to as the *time-drop*.) The only noted variation was in the high values of the time-drop for these apparently normal animals. But at the end of the experiment, one, a discard from certain staphylococcus experiments, was found to have multiple kidney abscesses, and the other coccidiosis hepatis.

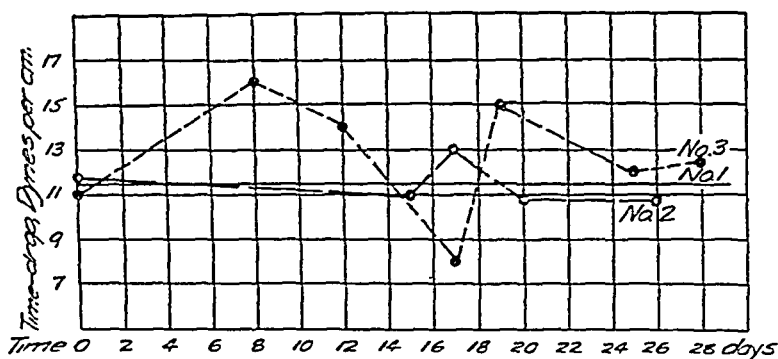
In the experiments on guinea pigs, a series of eight animals of about 200 gm. was sensitized with 0.1 cc. horse serum, intraperitoneal; another series of five was sensitized with 0.05 cc. horse serum, intravenous.

The critical concentration for giving the maximum time-drop values was found to be near that for the rabbit, 1/10,000, as found by du Noüy. This dilution was that used uniformly throughout the first part of this study, and the time-drop was for a 2 hour period.

⁴ An adequate criticism of the ring method of measurement of surface tension and a discussion of the difficulties of interpretation of the resulting data have recently appeared by Johlin, J. M., *J. Gen. Physiol.*, 1928, xi, 301.

An average of 46 readings of normal guinea pig sera, including the first readings of the test animals, gave a time-drop value of 11.5 dynes. When a series of four animals was carried through a period of 25 days, the variation from this average was not found to be more than 2 dynes in any given animal, with a maximum and minimum value difference of 4.2 dynes.

The series treated with 0.1 cc. horse serum showed a maximum increase of 7.6 dynes on the 7th day. (More frequent bleedings were avoided because of the possible effects of an induced anemia.) About the 16th day there was a fall below the normal level followed by a rise.



TEXT-FIG. 1. Surface tension changes in guinea pig serum:

1. Average time-drop of normal serum, 46 readings.
2. Variations in time-drop in a series of four normal animals carried through the same period as 3.
3. Variations in the time-drop in a series of seven sensitized animals.

For this fluctuation no explanation is offered. It is also to be noted in du Noüy's findings for rabbit immune serum.⁵ After the 25th day, the time-drop fell toward the normal level and remained at that point indefinitely—the average time-drop reading for this group being 12 dynes on the 45th day and 11.8 dynes on the 60th day after the sensitizing treatment. These changes are represented in Text-fig. 1.

In certain animals, the time-drop reached a value of 19 or more dynes, an increase of nearly 100 per cent of the initial reading.

⁵ du Noüy, P. L., *J. Exp. Med.*, 1923, xxxvii, 665.

The experiment was repeated with another series of five animals sensitized with 0.05 cc. horse serum, intravenous. A like result was obtained, although the variations from the normal were not so great. This may have been due to the following factors: the initial readings for the series were above normal, suggesting the effect of an obscure infection; the series was smaller; the sensitizing dose was smaller than in the first series, thereby reducing the toxic effect of the serum.

That the effects noted might be charged simply to changes resulting from the toxicity of a foreign serum for particular or various tissues was suggested and the following experiment was carried out: Four of the animals used in the group of normal controls were given a solution of 1 mg. of uranium nitrate subcutaneously. Three of the normal group were kept for controls.

Evidences of a severe infection appeared in the whole group by the 4th day, all the animals losing from 17 to 70 gm. in weight. The animals which had had uranium nitrate suffered the greatest loss of weight, appeared sicker, and two were found dead on the 8th day. The curve for the time-drop rose on the 8th day, but for both treated and untreated animals alike, as in the serum-treated series. This confirms du Noüy's findings for the effect of an epidemic—snuffles, for example, in rabbits. At any rate, the surface tension changes following an acute infection could not be said to be augmented by an added toxic agency.

Du Noüy showed in his study of immune serum in the rabbit that no further response in surface tension behavior could be elicited by a repetition of the treatment which had called out the first change. The question was suggested as to whether or not a treatment with a different antigen might occasion a comparable rise in the time-drop. Three of the series used in the earlier experiment to show the changes following horse serum treatment were given 0.05 cc. egg white, intraperitoneal. Two others were given uranium nitrate, as described. In no case was there an increase in the time-drop. One of the uranium nitrate animals died on the 8th day. All the others gained weight and remained normal in appearance.

CONCLUSIONS.

The change in surface tension behavior in the serum of sensitized guinea pigs is, as du Noüy has concluded for immunized rabbit serum, not referable to an antibody content, since we know that the capacity for transfer of sensitization remains in the serum indefinitely, while the increased time-drop phenomenon is a transitory manifestation. That this phenomenon cannot be invoked by a new antigen capable of calling out its specific antibody would seem to make this response one due to some basic stable alteration of a tissue active in the general process of sensitization. That this alteration is not one called out by such a simple toxic injury as a uranium nitrate nephritis is contributory evidence that the primary toxicity of the horse serum is not the specific factor involved.

SURFACE TENSION OF SERUM OF THE SENSITIZED GUINEA PIG.

II. SURFACE TENSION CHANGES IN THE BLOOD IN ANAPHYLACTIC SHOCK.

BY SUSAN GRIFFITH RAMSDELL.

(From the Research Institute of Cutaneous Medicine, Philadelphia.)

(Received for publication, March 5, 1928.)

Certain workers in Belgium and France have studied the surface tension of guinea pig serum in sensitization and shock, using a modification of the drop method.¹⁻⁷ They found, in general, that there is a lowering of the dynamic value of the surface tension whenever the clinical symptoms of shock are manifest.

In a tentative trial to corroborate these findings, using a technic measuring the static value of surface tension in dilutions of serum of guinea pigs in anaphylactic shock, we could not find appreciable variations from the normal or preshock values.

This failure suggested the use of whole blood for a comparative study by which the delay in measurement, and the influence therefrom of any progressive changes inaugurated in shock, might be avoided. This was found to be a practical procedure if the blood was transferred directly from the syringe to an amount of saline sufficient to inhibit clotting. In addition to static values, such preparation might be expected to show any inherent differences in the rapidity of a change in surface tension values, thereby giving different types of curves as a function of time.

¹ Zunz, E., and la Barre, J., *Compt. rend. Soc. biol.*, 1925, xcii, 223.

² Zunz, E., and la Barre, J., *Compt. rend. Soc. biol.*, 1924, xc, 118, 659.

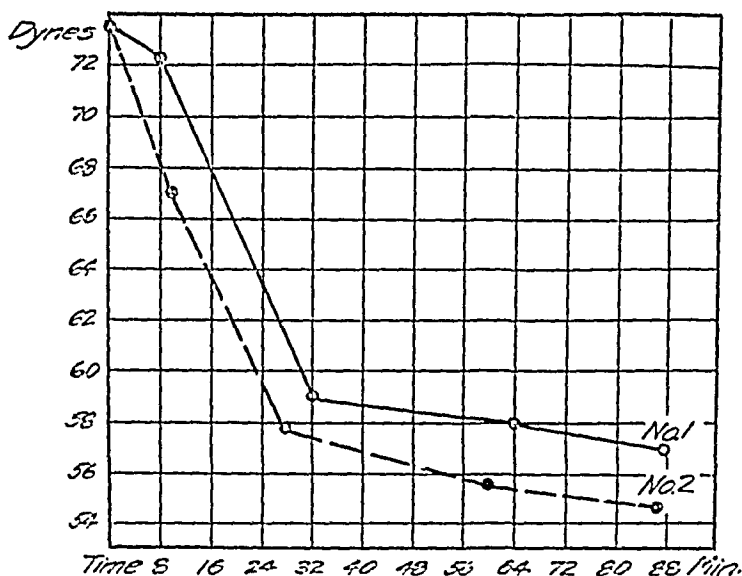
³ Zunz, E., and la Barre, J., *Compt. rend. Soc. biol.*, 1926, xcv, 858.

⁴ Zunz, E., *Bull. Acad. roy. m'd. Belgique*, 1925, v, 334.

⁵ Kopaczewski, W., *Compt. rend. Soc. biol.*, 1919, lxxxii, 590.

⁶ Kopaczewski, W., *Arch. phys. biol.*, 1922, ii, 1.

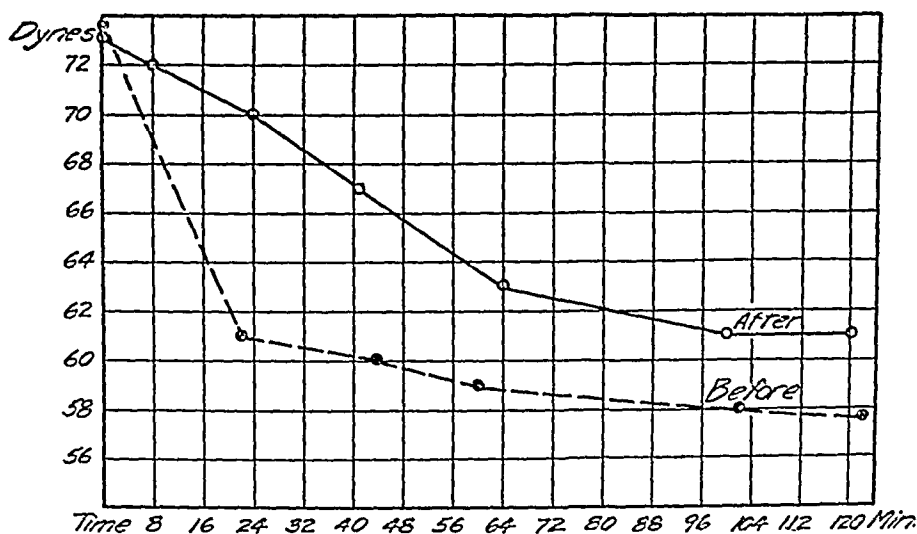
⁷ Zunz, E., and la Barre, *Arch. internat. physiol.*, 1923, xxi, 361.



TEXT-FIG. 1. Surface tension readings of normal guinea pig serum.

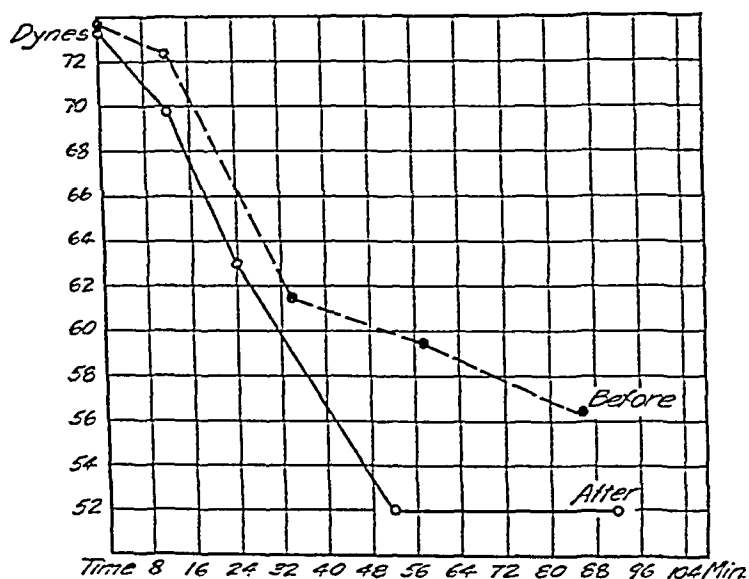
1. Before an injection of horse serum, 0.5 cc.

2. About 1 minute after.



TEXT-FIG. 2. Surface tension readings before and after immediate fatal anaphylactic shock.

A preliminary study was made on the serum of an immunized rabbit. The critical concentration for the maximum 2 hour time-drop value was found to lie around $1/5,000$. But it was found that such a whole blood dilution gave a static value reading lower than a serum dilution of $1/10,000$ by about 5 dynes, and that the solutions gave more regular values; the time-drop in the same rabbit's serum on 3 different days showing less than a dyne variation. Following an attempt at shock in the same animal, where the only sign of a dis-



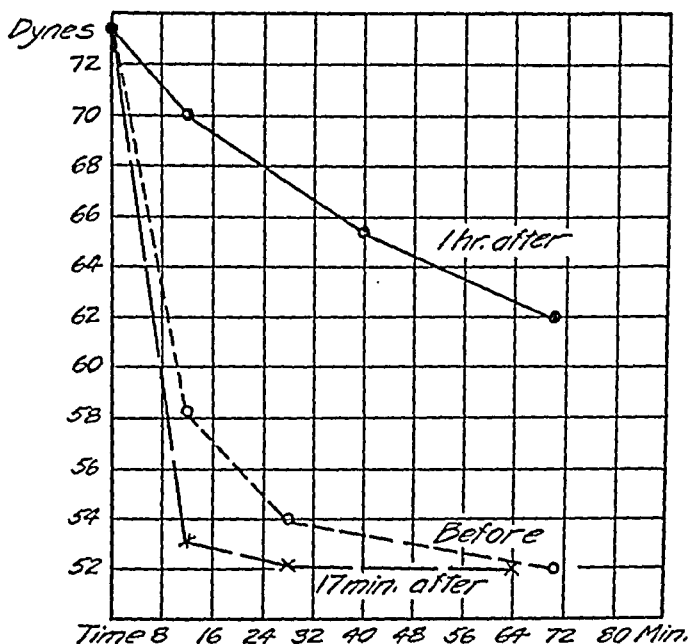
TEXT-FIG. 3. Surface tension readings before and after fatal, but not immediate, shock.

turbance was a heavy drowsiness, the static value reading was lowered by 3 dynes.

When a careful, repeated titration for a critical concentration for normal guinea pig whole blood was made, the lowest static values were found to occur around $1/8,000$ with a second low value around a dilution just greater than $1/10,000$. The former concentration was chosen for the ensuing study. That this does not represent serum alone is shown by the difference in static surface tension values for

the same dilution—lower by 7 dynes for blood than serum alone. A similar change in the critical concentration was noted by du Noüy for rabbit plasma.⁸

In preparation for these experiments a special collection of horse serum was made with all the precautions as to glassware that were taken in the general work. Eleven animals remaining from the study of sensitized serum, with a sensitization period of 60 to 90 days, and



TEXT-FIG. 4. Effect of cholesterol, 0.5 to 1.0 mg., intravenous, upon surface tension readings.

two others with a 10 day period, together with twelve normal animals were used for the experiment.

The effect of an intravenous injection of horse serum into normal guinea pigs gave a fall in the time-drop of about 2 dynes. Comparative curves for such a normal are shown in Text-fig. 1.

In case of shock, there was a considerable variation in values—from a rise in one case of a 5 dyne time-drop, to a fall of like value. Where

⁸ du Noüy, P. L., *J. Exp. Med.*, 1927, xlv, 1.

there is variation in the factors of (1) size of dose, (2) of time-interval between injection of serum and withdrawal of test blood, (3) of time-interval between injection of serum and onset of shock symptoms, such irregular findings are not surprising. In general, it can be shown that the first response to a dose of homologous antigen is a rapid fall in surface tension, and that this is greater in direct proportion to the degree of reaction as shown in the usual anaphylactic manifestations, or to the time elapsing between the injection of the serum and the withdrawal of blood.

In cases where the sensitized animal failed to show signs of shock, or where death occurred immediately after this dosage (less than 1 minute), or where death was delayed for several minutes, the curves representing the surface tension readings before and after the serum injection were not markedly different from those shown by the normal animal after such injection; but in certain cases the curves for the postinjection blood followed a higher level. In these, the differences in the curves tended to disappear after about 60 minutes, but the relative positions of the levels did not change (Text-fig. 2). When death is delayed for more than 1 minute and blood is taken after full development of shock symptoms, a definite lowering occurs in the resulting curves (Text-fig. 3).

The divergent variations in findings are not surprising when one reflects upon the very complex mixtures with which one deals and upon the antagonistic behavior of such colloidal solutions—this influenced, in turn, by relative concentrations, as shown by du Noüy for serum solutions and sodium oleate. Such active substances as cholesterol and lipoids have been shown to be increased in the blood following shock.^{9,10}

This was verified as to an increase amounting to 16 per cent in cholesterol content of whole blood following shock in the guinea pig. A corresponding active effect upon the blood of a normal guinea pig was shown (Text-fig. 4) following the intravenous injection of a suspension of 1 mg. of cholesterol. *In vitro* a fall was followed by a rise when the cholesterol was used in a dilution of 1/20,000. When

⁹ Zanz, E., and la Barre, J., *Compt. rend. Soc. biol.*, 1925, xciii, 1044.

¹⁰ Van de Velde, J., *Compt. rend. Soc. biol.*, 1927, xcvi, 1825.

powdered cholesterol was added directly to the serum dilution, a fall of 11 dynes occurred at once, and recovery did not take place. The surface tension of a mixture of saline and powdered cholesterol was lowered by 22 dynes.

In a few experiments with histamine shock, the histamine alone did not lower the surface tension of saline, but did lower that of a serum solution *in vitro*. When the blood was examined after shock, the same serum might exhibit a rise in surface tension following a sublethal dose, and a return to normal at a later period, or a marked fall following a dose sufficient to bring on an immediate and fatal shock. We are here probably concerned with the behavior of serum plus histamine, and each of these or their reaction product with liberated tissue substances (glycogen, cholesterol, etc.), where the effect may be primarily additive and secondarily antagonistic.

CONCLUSION.

The primary change in surface tension of serum incident to anaphylactic shock is probably due to a lowering of the surface tension of the serum by the addition of the antigen serum. But this may be followed by a further decrease or by an increase depending on the intensity and duration of certain secondary tissue changes.

THE PATHOGENESIS OF EARLY OBSTRUCTIVE JAUNDICE.

By E. S. GUZMAN BARRON, M.D., AND JOHN H. BUMSTEAD, M.D.

(From the Chemical Division of the Medical Clinic and the Department of Pathology, the Johns Hopkins University, Baltimore.)

(Received for publication, March 21, 1928.)

INTRODUCTION.

For many years investigators have been concerned with the question of the mechanism of early obstructive jaundice, some studying the process as a whole, others limiting themselves to certain aspects of the problem.

In cases of complete obstructive jaundice of long standing, the mechanism is fairly well understood. As shown by Eppinger (1) for the first time, and confirmed by Abramow and Samoilowicz (2), the bile canaliculi in these cases are distended and tortuous and their walls frequently ruptured, the bile emptying into the pericapillary lymph spaces. These observations lead to the theory of increased tension of the bile capillaries due to bile continually flowing from the liver cells but unable to reach the duodenum. As a result of increased tension the vessel walls rupture and the extravasated bile is absorbed either by the blood capillaries or by the lymphatics. It then finds its way into the blood stream and is partly excreted in the urine and partly deposited in the tissues. This theory however, especially in the initial stage of the process, has found many opponents.

Minkowski, as early as 1892 (3) and again in 1904 (4), emphasizes the importance of alterations of the liver cells in causing jaundice and proposes his theory of "*paracholie*" or the passage of bile from the pathological liver cells directly into the perilymphatic spaces, rather than into the bile capillaries. Sterling (5) some years later arrived at the same conclusion. He was not able to observe rupture of the bile capillaries after biliary obstruction and laid emphasis on early changes in the liver cells themselves.

Ogata (6) says that before the rupture of the bile capillaries there is a dilatation due to increased pressure. He believes that a necrosis of liver cells appears in the first stages of biliary obstruction. Browicz (7), Jagie (8), Kodama (9) and Hiyeda (10) have confirmed these findings. They were unable to observe any rupture of bile capillaries in early obstructive jaundice. The last two investigators, studying liver sections from 7 to 11 hours after ligation of the ductus choledochus, could

observe only a dilatation of bile capillaries, and in some cases the formation of bile thrombi ("Gallenthromben").

We must discard the early observations regarding the time of first appearance of bile in the blood stream because of faulty technique. Either the Gmelin test was used to detect the bile pigments or else the investigators tested for bile salts. We know now that the Gmelin test is the least sensitive of all bilirubin reactions, and even as yet we have no method sensitive enough to detect small traces of bile salts in blood or urine.

Eppinger (11) in 1920 stated that 24 hours after obstruction one can notice an increase in the bilirubin content of the blood. Lepehne (12) in 1921, experimenting with rabbits and employing the Van den Bergh test to detect bilirubin, observed in 3 out of 5 animals an increase of blood bilirubin from 0.4 to 6.5 units within 24 hours. Bloom (13), using dogs previously nephrectomised, noticed the presence of the indirect Van den Bergh reaction from 1 hour and 19 minutes to 4 hours after ligation of the bile ducts. Kodama (9) in 1925 observed, in rabbits and dogs, the appearance of the indirect reaction 7 hours after ligation of the ductus choledochus, the direct reaction appearing 10 hours after obstruction. More recently Bollmann, Sheard and Mann (14) employing the Keuffel and Esser spectrophotometer to detect the bilirubin showed that the increase of bilirubin in the blood begins 5 minutes after ligation of the bile ducts. They further studied the pressure within the bile ducts and they observed that it was raised from 14 mm. water pressure to 270 or 300 mm. after obstruction. In conclusion they state: "As soon as the pressure in the biliary ducts has risen to 250 or 300 mm. of water, the hepatic cells become impervious to bilirubin so that bile pigment is neither excreted into the bile capillaries nor is it absorbed from the blood by the hepatic cell."

Report of Experiments.

I. First Appearance of Bile in the Blood Stream after Biliary Obstruction.

In all of our experiments we have employed dogs previously kept in metabolic cages and known not to show the so called physiologic bilirubinuria.

The method employed to detect bilirubin in the blood and lymph has been the well known Van den Bergh test, the reactions obtained being divided into three groups:

Direct Reaction.—Instantaneous appearance of the colour reaction after the addition of the reagent, reaching its maximal intensity in from 20 to 30 seconds.

Biphasic Reaction.—(Combination of direct and indirect reaction) in which the colour reaction begins within the first 30 seconds but does not reach its maximum intensity until from 1 to 30 minutes later.

Indirect Reaction.—In which the colour reaction begins from 1 to 3 minutes or more after the addition of the reagent, requiring sometimes the addition of alcohol. The maximum intensity is reacted after a variable time and the action of alcohol is essential to produce the maximum colour.

We used according to Lepehne's technique (15) 0.25 cc. of lymph or serum to which was added 0.20 cc. of freshly prepared reagent. The quantitative estimation was made with the Bausch and Lomb microcolorimeter, with, as standard, a solution of crystallised cobalt sulfate¹ previously checked against a solution of pure bilirubin. The determinations in serum giving the indirect Van den Bergh reactions were made according to Van den Bergh's technique, and the quantitative estimations in those giving biphasic or direct reactions were made by the modification of Thannhauser and Andersen (16).

The operative technique was the same as that employed by Bloom. All the dogs were given a preparatory injection of morphine followed in 30 minutes by ether anesthesia and were kept lightly under the anesthetic until the end of the experiment.

In this series of experiments, both kidneys were removed and the ductus choledochus and cystic duct ligated through a midline abdominal incision. Samples of blood were taken every 15 minutes from the carotid artery into which a cannula had been previously introduced. When the direct Van den Bergh reaction appeared in the blood the dogs were killed with ether. At necropsy the ligatures were verified and samples of liver taken for microscopic examination.

Five experiments were performed; all gave concordant results. The following protocol gives the details in one experiment; the results of the series have been summarised in Table II.

Protocol of Experiment on Dog 8.

Female, weight 11.5 kilos. At 10 a.m. a hypodermic injection of morphine was given. At 10:30 a.m. anesthesia with ether. At 10:50 a.m. a cannula was put in the carotid artery and a sample of blood taken. At 11:15 a.m. midline abdominal incision and transperitoneal double nephrectomy. At 11:30 a.m. ligation of ductus choledochus and cystic duct. Samples of liver were taken every 15 minutes.

The experiment lasted 6 hours and 20 minutes. The dog was killed with ether and a necropsy performed. The ligatures were well placed; there was no blood in the abdominal cavity. The liver was slightly hyperemic; on section, the portal and interlobular veins were found to be full of blood; the bile ducts were full of bile. The lymph vessels looked distended and greenish in colour. The spleen was

¹ 3.915 gm. of $\text{CoSO}_4 \cdot 7\text{H}_2\text{O}$ in 100 cc. distilled water is equivalent to 0.005 mg. per cc.

full of blood but not swollen. Samples of liver were taken for microscopical sections.

At 1 p.m., that is 1 hour and 30 minutes after biliary obstruction, the blood serum gave the indirect Van den Bergh reaction, which remained until 2:15. At this time, that is 2 hours and 45 minutes after obstruction, the Van den Bergh reaction became biphasic. At 4:15 p.m. or 4 hours and 45 minutes after obstruction, the reaction became direct rising steadily until the animal was killed.

TABLE I.

Dog 8. Experimental Obstructive Jaundice in Dogs Previously Nephrectomised. Van den Bergh Reaction and Bilirubin Estimation in the Blood Serum, Withdrawn Every 15 Minutes from the Carotid Artery.

Blood	Van den Bergh reaction			Time after obstruction	Quantitative estimation of bilirubin
Time	Direct	Biphasic	Indirect		
<i>a.m.</i>					<i>mg. per liter</i>
10:50	—	—	—		
11:30	Ligation of common and cystic bile ducts				
11:45					
12 m.	—	—	—		
<i>p.m.</i>					
12:15	—	—	—		
12:30	—	—	—		
12:45	—	—	—		
1	—	—	+	1 hr., 30 min.	Traces
1:15	—	—	+		"
1:30	—	—	+		"
1:50	—	—	+		"
2:15	—	+		2 hrs., 45 min.	1.50
2:35	—	+			2.00
2:55	—	+			2.50
3:15	—	+			3.50
3:35	—	+		4 hrs., 45 min.	3.75
4:15	+				4.00
4:35	+				4.50
4:55	+				5.50

In Table II we summarise the five experiments performed. We see that in the early hours after biliary obstruction in dogs, the time of appearance of bilirubin in the blood is quite constant. The indirect reaction appears during the 2nd hour after obstruction is performed and persists from 1 hour to 1 hour and 45 minutes. The biphasic reac-

tion then appears and lasts from 1 hour and 10 minutes to 2 hours and 10 minutes. Finally the direct reaction appears 4 to 5 hours after obstruction and will last as long as the obstruction persists.

The behaviour of the biphasic reaction was not always the same. Thus in Dog 7 the biphasic reaction first appeared 2 hours and 30 minutes after obstruction; in the following sample, 15 minutes later, the reaction was indirect; in the sample collected 30 minutes later it had become biphasic again.

In Fig. 1 we give the results of the quantitative estimations of bilirubin made in the five experiments. The rise in the bilirubin content of the blood, after it is first detected, is regular until the end of the

TABLE II.

Experimental Obstructive Jaundice in Nephrectomised Dogs. Summary of Experiments Showing the Time of Appearance of Van den Bergh Reaction in the Blood Serum.

Dog No.	Sex	Weight kg.	Time of appearance of the Van den Bergh reaction in the blood serum, after total biliary obstruction		
			Indirect	Biphasic	Direct
3	F.	8	1 hr., 25 min.	2 hrs., 15 min.	4 hrs., 23 min.
4	F.	12.2	1 hr., 15 min.	3 hrs.	4 hrs., 10 min.
6	M.	10	1 hr., 20 min.	2 hrs., 40 min.	4 hrs.
7	F.	5.4	1 hr., 20 min.	2 hrs., 30 min.	4 hrs., 55 min.
8	F.	11.5	1 hr., 30 min.	2 hrs., 45 min.	4 hrs., 45 min.

experiment. We may add that another series of experiments now being performed indicates that the rise is continuous and slow until 40 hours after total biliary obstruction. Then a sudden rise occurs which is maintained at more or less the same level until relief of the obstruction or until death. We take from these experiments, one shown in Fig. 2, which indicates the bilirubin content in the blood during the whole period of obstruction until relieved by a biliary fistula. The blood bilirubin after relieving the biliary obstruction, behaves, as we see in this figure, in a manner exactly the reverse of that noted in early obstructive jaundice. The 3rd day after relief of the obstruction the blood bilirubin, besides diminishing in quantity, has become

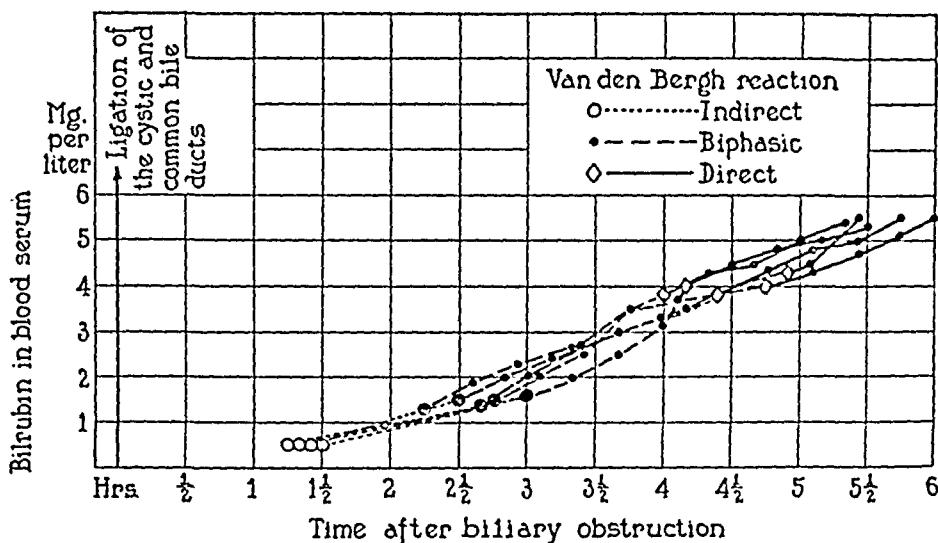


FIG. 1. Experimental obstructive jaundice in nephrectomised dogs. Curve of blood bilirubin and Van den Bergh reaction in early obstructive jaundice.

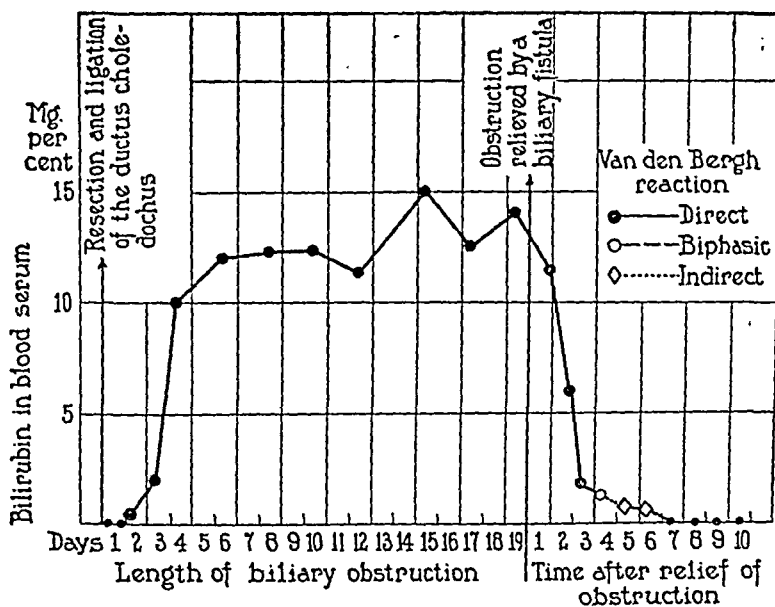


FIG. 2. Experimental obstructive jaundice in dogs. Curve of blood bilirubin during the whole period of obstruction till 7 days after relief of obstruction by a biliary fistula.

biphasic in type; on the 5th day the Van den Bergh reaction has changed into an indirect type. On the 7th day after relief of the obstruction, the bilirubin had disappeared from the blood.

II. Route Taken by the Bile to Reach the Blood Stream after Biliary Obstruction.

The question of the route taken by the bile to reach the blood stream after complete biliary obstruction has also attracted considerable attention. Many investigators have supposed the escape of bile from the liver in obstructive jaundice to be due to the activity of the lymphatic apparatus, while others uphold the view that this process is essentially an absorption of bile from the liver by the activity of the blood capillaries.

Fleischl (17) after ligating the ductus choledochus collected the lymph through a fistula made in the thoracic duct and found it charged with bile, while after 5 hours he could not detect any bile in the blood stream. Kunkel (18) and Kufferath (19) reached the same conclusion from determinations of the bile salts. Harley (20) ligated the common bile duct of two dogs, and in one in addition closed the thoracic duct. He observed in the dog with thoracic duct intact, that bile appeared in the urine in the course of a few hours after biliary obstruction, while in the dog with thoracic duct closed as long a period as 8 days elapsed before there was any such discharge. Wertheimer and Lépage (21) performed a series of experiments on dogs with a fistula of the thoracic duct and ligation of the ductus choledochus. They injected ox bile into the bile duct of the right hepatic lobe, and after a certain time could observe the presence of the cholehematin spectrum in the bile coming from the left hepatic lobe, but in these experiments they could not look for the presence of cholehematin in lymph, because, "quand le spectre de la cholehematine commençait à se montrer dans la bile du chien, la lymphe était devenue rouge." They then injected solutions of pure bilirubin under pressure and observed the appearance of the Gmelin reaction in the lymph 1 hour after the injection and its appearance in the urine 2 hours and 30 minutes after the injection. They conclude: "La vérité est que les deux ordres des vaisseaux contribuent à leur resorption et. . . . Le rôle des lymphatiques n'en reste pas moins important." But 1 year later (22) these same investigators performed simultaneous ligations of the ductus choledochus and thoracic duct and observed the appearance of bile in the urine. They considered the lymphatic route to be of secondary importance because they observed that bile pigment also appeared in the urine in a like manner when they ligated the ductus choledochus only. Mendel and Underhill (23) injected indigo carmine, KI, K ferrocyanide, milk and I, and milk into the common bile duct, and observed that these foreign substances appeared first in the urine

and then in the lymph with the exception of the milk-iodine mixture, which was detected 65 minutes after the injection in the lymph but not in the urine. They concluded that the hepatic capillaries are the important factor in this absorption. Whipple and King (24) ligated the common bile duct, exposed the thoracic duct and placed a ligature at its junction with the jugular vein. They detected the bile pigments in urine and lymph by the use of the Salkowski test. From their experiments they concluded that in obstructive jaundice the bile which escapes from the

TABLE III.

Dog 11. Experimental Obstructive Jaundice in Nephrectomised Dogs. Van den Bergh Reaction and Bilirubin Estimation in Samples of Lymph Withdrawn Every 20 Minutes from a Thoracic Duct Fistula, Previously Performed.

Lymph	Van den Bergh reaction			Time after obstruction	Quantitative estimation of bilirubin
Time	Direct	Biphasic	Indirect		
12:30	—	—	—	1 hr.	mg. per liter
12:40	Ligation of common and cystic bile ducts				
1:00	—	—	—		
1:20	—	—	—		
1:40	—	—	+		
2:00	—	—	+		
2:20	—	—	+		
2:40	—	—	+		
3:00	—	—	+		
3:20	—	+		2 hrs., 40 min.	Traces
3:40	—	+			
4:00	—	+			
4:20	—	+			
4:40	—	+		4 hrs., 40 min.	0.5
5:00	—	+			
5:20	—	+			
5:40	+				
6:00	+				
6:15	+				

liver is absorbed by the blood capillaries and carried by the blood to the kidneys: "at best the lymphatic system is a secondary factor in the mechanism of jaundice." Bloom in the paper previously mentioned (13) detected bilirubin in blood and lymph by the Van den Bergh technique and observed that a positive test for bilirubin was obtained in the lymph while the blood was still pigment-free. He concluded that "during the first hours after obstruction of the common bile duct, the bile pigments are carried from the liver by the lymph stream and by it are emptied into the general circulation."

Report of Experiments.

The purpose of our second series of experiments was to study the route taken by the direct bilirubin found some hours after obstruction, to reach the blood stream.

The protocol of one experiment is given in detail; the results of the series of experiments have been summarised in Tables III and IV.

TABLE IV.

Dog 11. Experimental Obstructive Jaundice in Nephrectomised Dogs. Van den Bergh Reaction and Bilirubin Estimations in Samples of Blood Serum Withdrawn Every 20 Minutes from the Carotid Artery.

Blood	Van den Bergh reaction			Time after obstruction	Quantitative estimation of bilirubin
Time	Direct	Biphasic	Indirect		
12:20	—	—	—		mg. per liter
12:40	Ligation of common and cystic bile ducts				
1:05	—	—	—		
1:20	—	—	—		
1:40	—	—	—		
2:00	—	—	—		
2:20	—	—	—		
2:40	—	—	—		
3:00	—	—	—		
3:20	—	—	+	2 hrs., 40 min.	Traces
3:40	—	—	+		Traces
4:00	—	—	+		0.5
4:20	—	—	+		0.8
4:40	—	+		4 hrs.	1.00
5:00	—	+			1.30
5:20	—	+			1.50
5:40	—	+			1.50
6:00	+			5 hrs., 20 min.	2.00
6:15	+				2.00

Dog 11.—Male, weight 16 kilos. An injection of 3 cg. of morphine was given 39 minutes before ether anesthesia was begun. All the veins entering the left external jugular vein at its junction with the thoracic duct were ligated and a cannula placed in the jugular vein, at 12:15 p.m. The lymph flow was regular at a rate of 20 cc. to 30 cc. per hour. At 12:20 p.m. a cannula was inserted in the right carotid artery and a sample of blood withdrawn. At 12:32 p.m. midline incision and transperitoneal double nephrectomy. At 12:40 p.m. ligation of common and

cystic bile ducts. Samples of blood were withdrawn every 20 minutes, the lymph being examined at the same time. The indirect Van den Bergh reaction appeared first in the lymph 1 hour after the obstruction. 2 hours and 40 minutes after the obstruction the Van den Bergh reaction in the lymph became biphasic, while the indirect reaction appeared in the blood serum. 4 hours and 40 minutes after obstruction the Van den Bergh became direct in the lymph, and 40 minutes later, that is 5 hours and 20 minutes after biliary obstruction, the blood serum showed also the direct Van den Bergh reaction. The dog was killed at 6:20 p.m. with ether. At necropsy the ligatures of bile ducts were well placed. The liver appeared hyperemic. On section the bile ducts were distended with bile. The lymphatics were distended and greenish in colour. Samples of liver were taken for microscopic examinations.

TABLE V.

Experimental Obstructive Jaundice in Dogs Previously Nephrectomised. Ligation of Bile Ducts and Thoracic Duct Fistula.

Dog No.	Sex	Weight kg.	Time of appearance of the Van den Bergh reaction after biliary obstruction in dogs with thoracic duct fistula					
			Lymph			Blood		
			Direct	Indirect	Biphasic	Direct	Indirect	Biphasic
11	M.	16	4 hrs., 40 min.	1 hr.	2 hrs., 40 min.	5 hrs., 20 min.	2 hrs., 40 min.	4 hrs.
14	F.	12.2	4 hrs., 20 min.	50 min.	2 hrs., 25 min.	5 hrs., 15 min.	2 hrs. 30 min.	4 hrs., 15 min.
17	F.	14.4	4 hrs., 50 min.	1 hr.	2 hrs., 50 min.	5 hrs., 45 min.	2 hrs., 55 min.	4 hrs., 30 min.

In one additional experiment the thoracic duct was ligated accidentally. We proceeded as previously, taking samples of blood every 15 minutes. In this dog, the indirect Van den Bergh first appeared in the blood 2 hours and 45 minutes after obstruction. The biphasic reaction appeared 4 hours and 30 minutes and the direct reaction 5 hours and 30 minutes after obstruction. The Van den Bergh reaction in this case behaved as it did in the experiments with thoracic duct fistula.

We summarise in Table V the results of our three experiments, which are in close agreement.

We see from these experiments that after complete biliary obstruc-

tion associated with a thoracic duct fistula, the bile appears first in the thoracic duct before it is detectable in the blood, but that 1 hour later it is present in the blood, not only when the lymph is being drained from the thoracic duct, but even when the duct has been ligated.

Microscopic Examinations of the Liver of These Dogs, 5 to 6 Hours after Total Biliary Obstruction.

It is generally agreed that rupture of bile capillaries appears only in the late stages of biliary obstruction. We made microscopical examinations of the liver of the dogs reported in our experiments. Hematoxylin and eosin sections showed no atrophy or necrosis of the liver cells which appeared quite normal. The bile ducts were not noticeably distended and no bile thrombi were observed. The Kupffer cells in the liver of Dog 8 (whose protocol has been given) contained a moderate amount of brown pigment which could be stained for iron. No such pigment was found in any of the others. Frozen sections stained with Scharlach R revealed only a slight amount of finely divided fat in the liver cells. The cells lining the smaller bile ducts consistently contained fat droplets of various size. Sections were fixed and stained by the method described by Vance (25) for the demonstration of bile canaliculi. The canaliculi were well stained, prominent and slightly distended but the most careful search failed to reveal the rupture of any of them into adjacent lymph spaces.

DISCUSSION.

Taking first in consideration the behaviour of the Van den Bergh reaction in early obstructive jaundice, we see from our experiments that the indirect reaction appears first, then the biphasic and some hours later the direct reaction, which remains during the whole period of obstruction. In spite of the many investigations carried out concerning the nature of the Van den Bergh reaction, it still remains an unsettled problem. Nevertheless from McNee's (26) and Van den Bergh's (27) researches corroborated by Andrews' important work (28), it is clear that the bilirubin normally present in the circulating blood gives only the indirect reaction, and that on passing through the liver cell during excretion, it is changed in some as yet undeter-

mined manner into a form which gives the direct reaction.² What is the significance of the indirect reaction which uniformly appears in the plasma of this animal soon after obstruction, and lasts a short time? It seems to us that a possible explanation for this phenomenon would be that as a consequence of the biliary obstruction a nervous reflex may occur which would lead to sudden paralysis of the function of the liver cells and as a result the circulating bilirubin would remain in the blood stream. This would be analogous to the temporary reflex paralysis of the excretory function of the kidney after ligation of the ureters, which is a well known phenomenon. As the process of bilirubin formation goes on, the bilirubin would soon reach a level sufficient to give a positive test with the diazo reagent. This reflex phenomenon would not last very long. Some hours after obstruction the liver cells would resume their function and the bilirubin giving the indirect reaction would be changed by them into bilirubin giving the direct reaction. We are at present engaged in a study of this possibility.

Another difficult aspect of the problem now presents itself. If in the first hours of obstructive jaundice there is no rupture of bile canaliculi, by what process does the direct reacting bile appear in the blood? In short what is the intimate mechanism of early obstructive jaundice? This is a field in which suggestions only can be made. Must we return to Minkowski's theory as offering the only plausible explanation? The double polarization of the liver cells has been known since Claude Bernard's memorable discovery of the glycogenic function of the liver (29). We know that the epithelial liver cell can function in two different ways: as a gland of internal secretion expelling its products into the blood stream and as a gland of external secretion excreting bile into the bile canaliculi. But it is extremely difficult to comprehend by what mechanism the excretion of bile can shift from an external to an internal secretion.

A close examination of the microscopic sections of the liver in early obstructive jaundice, stained by Vance's technique, shows the bile canaliculi dilated and extending between the liver cells in small distended pouches, the blind end of these lying in contact with the

² In the dog, bilirubin is present in the plasma normally in amounts too small to be detected by the Van den Bergh test.

pericapillary spaces. It would be quite easy to believe that bile might diffuse from these thin walled pouches into the tissue spaces without any actual rupture of the bile canaliculi. This diffusion would be favored by the mounting pressure inside the bile ducts, which rises rapidly according to Bollmann, Sheard and Mann (14).

Regarding the second question, that of the route taken by the bile to reach the blood, since, as we have seen in the thoracic duct fistula experiments, direct reacting bilirubin soon finds its way into the perivascular lymph spaces which surround the hepatic capillaries, bile may diffuse into these capillaries and thus directly enter the blood stream, as we have observed in our experiments.

We think our experiments explain the contradictory results of other investigators concerning the route by which bilirubin reaches the blood in obstructive jaundice. We feel justified in concluding as Wertheimer and Lépage did after their first observations: "*La vérité est que les deux ordres des vaisseaux contribuent à leur resorption et . . . Le rôle des lymphatiques n'en reste pas moins important.*"

CONCLUSIONS.

1. After experimental ligation of the bile ducts in dogs, two distinct processes are clearly manifested: first, the accumulation of the normally circulating bilirubin in the blood with its characteristic indirect Van den Bergh reaction for a period of several hours, and second, the subsequent appearance of the bile bilirubin giving the direct Van den Bergh reaction. It is possible that the first process may be due to a temporary reflex inhibition of the function of the liver cells due to ligation of the duct and comparable to the same phenomenon which usually occurs in the kidney when the ureter is ligated. The second process begins before any rupture of the bile capillaries is visible. Liver sections made 6 to 7 hours after obstruction show these bile capillaries dilated and extending between the liver cells in small distended pouches the blind end of these lying in contact with the pericapillary spaces. It is possible that bile may diffuse from these thin walled pouches into the perivascular lymph spaces, this diffusion being favored by the mounting pressure inside the bile ducts.

2. In early obstructive jaundice bile first appears in the lymph, but exclusion of the thoracic duct from the circulation by drainage causes

a delay of only a few hours in the appearance of bile bilirubin in the blood stream. We must therefore conclude that after biliary obstruction bile enters the circulation both by way of the blood capillaries and the lymphatics, although the latter route is the more important.

We wish to express our thanks to Dr. Arnold R. Rich for his constant assistance and many valuable suggestions.

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PHOTOSENSITIZATION OF ANIMALS AFTER THE INGESTION OF BUCKWHEAT.

By CHARLES SHEARD, Ph.D., HAROLD D. CAYLOR, M.D., AND CARL SCHLOTTHAUER, D.V.M.

(From the Section on Physics and Biophysical Research, the Section on Surgical Pathology, and the Division of Experimental Pathology and Surgery, Mayo Clinic and The Mayo Foundation, Rochester, Minnesota.)

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It has been known for a long time that certain animals, following the ingestion of various substances, are rendered sensitive to certain types of radiant energy. The present study covers biologic and physical observations on the degree of sensitization, the character of the sensitizing light and the nature of the photodynamic substance of buckwheat. White rabbits, mice, rats, goats, swine, dogs and varicolored guinea pigs were studied. Sunlight, carbon arc lamp (Efka or Hoffman) with Conradty-Norris vacuum carbon electrodes and quartz mercury vapor arcs (Victor X-Ray Corporation), both with and without various filters, were employed for irradiation. All of these sources contain infra-red, visible and ultra-violet radiations.

RÉSUMÉ OF LITERATURE.

Considerable literature, especially European, has appeared on the subject of diseases in animals and man brought about by optical sensitization. Somewhat detailed accounts of the contributions relative to diseases due to exogenous sensitization are to be found in the brochures by Hausmann and by Mayer.

Years ago European stockmen found that in certain animals which had ingested buckwheat (plant or seed), erythema, itching, edema and convulsions developed and, in many cases, paralysis and death, on exposure to out-of-door sunshine. However, untoward symptoms did not arise if the animals were kept indoors or in partial darkness and they recovered from the sensitization induced by eating certain plants if they were removed from the light early in the onset of the symptoms. Likewise it was found that the non-pigmented areas of the body were affected readily, whereas the pigmented areas were kept intact. A relatively high degree of intensity of light appears to be a factor in all such diseases as so called buckwheat poisoning (fagopyrismus). Buckwheat causes sensitization to light

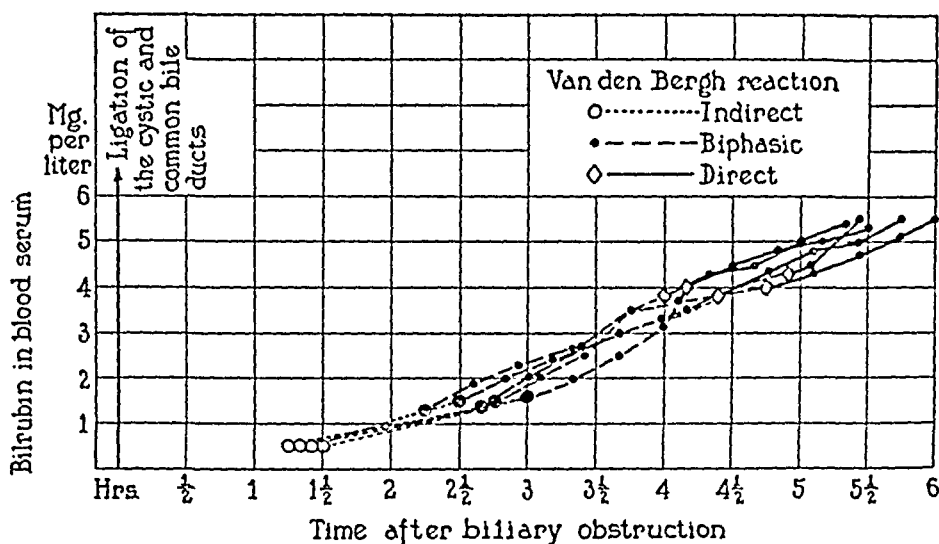


FIG. 1. Experimental obstructive jaundice in nephrectomised dogs. Curve of blood bilirubin and Van den Bergh reaction in early obstructive jaundice.

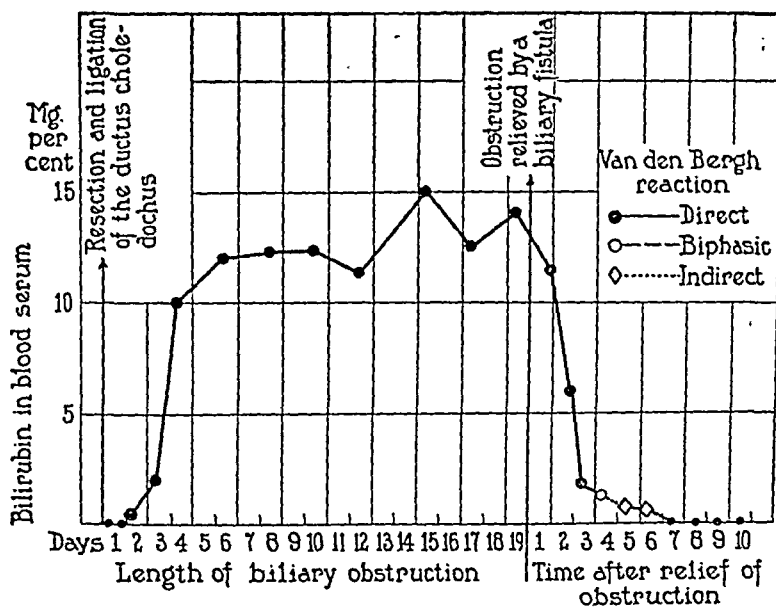


FIG. 2. Experimental obstructive jaundice in dogs. Curve of blood bilirubin during the whole period of obstruction till 7 days after relief of obstruction by a biliary fistula.

biphasic in type; on the 5th day the Van den Bergh reaction has changed into an indirect type. On the 7th day after relief of the obstruction, the bilirubin had disappeared from the blood.

II. Route Taken by the Bile to Reach the Blood Stream after Biliary Obstruction.

The question of the route taken by the bile to reach the blood stream after complete biliary obstruction has also attracted considerable attention. Many investigators have supposed the escape of bile from the liver in obstructive jaundice to be due to the activity of the lymphatic apparatus, while others uphold the view that this process is essentially an absorption of bile from the liver by the activity of the blood capillaries.

Fleischl (17) after ligating the ductus choledochus collected the lymph through a fistula made in the thoracic duct and found it charged with bile, while after 5 hours he could not detect any bile in the blood stream. Kunkel (18) and Kufferath (19) reached the same conclusion from determinations of the bile salts. Harley (20) ligated the common bile duct of two dogs, and in one in addition closed the thoracic duct. He observed in the dog with thoracic duct intact, that bile appeared in the urine in the course of a few hours after biliary obstruction, while in the dog with thoracic duct closed as long a period as 8 days elapsed before there was any such discharge. Wertheimer and Lépage (21) performed a series of experiments on dogs with a fistula of the thoracic duct and ligation of the ductus choledochus. They injected ox bile into the bile duct of the right hepatic lobe, and after a certain time could observe the presence of the cholehematin spectrum in the bile coming from the left hepatic lobe, but in these experiments they could not look for the presence of cholehematin in lymph, because, "quand le spectre de la cholehematine commençait à se montrer dans la bile du chien, la lymphe était devenue rouge." They then injected solutions of pure bilirubin under pressure and observed the appearance of the Gmelin reaction in the lymph 1 hour after the injection and its appearance in the urine 2 hours and 30 minutes after the injection. They conclude: "La vérité est que les deux ordres des vaisseaux contribuent à leur resorption et. . . . Le rôle des lymphatiques n'en reste pas moins important." But 1 year later (22) these same investigators performed simultaneous ligations of the ductus choledochus and thoracic duct and observed the appearance of bile in the urine. They considered the lymphatic route to be of secondary importance because they observed that bile pigment also appeared in the urine in a like manner when they ligated the ductus choledochus only. Mendel and Underhill (23) injected indigo carmine, KI, K ferrocyanide, milk and I, and milk into the common bile duct, and observed that these foreign substances appeared first in the urine

and then in the lymph with the exception of the milk-iodine mixture, which was detected 65 minutes after the injection in the lymph but not in the urine. They concluded that the hepatic capillaries are the important factor in this absorption. Whipple and King (24) ligated the common bile duct, exposed the thoracic duct and placed a ligature at its junction with the jugular vein. They detected the bile pigments in urine and lymph by the use of the Salkowski test. From their experiments they concluded that in obstructive jaundice the bile which escapes from the

TABLE III.

Dog 11. Experimental Obstructive Jaundice in Nephrectomised Dogs. Van den Bergh Reaction and Bilirubin Estimation in Samples of Lymph Withdrawn Every 20 Minutes from a Thoracic Duct Fistula, Previously Performed.

Lymph	Van den Bergh reaction			Time after obstruction	Quantitative estimation of bilirubin
Time	Direct	Biphasic	Indirect		
12:30	—	—	—	1 hr.	mg. per liter
12:40	Ligation of common and cystic bile ducts				
1:00	—	—	—		
1:20	—	—	—		
1:40	—	—	+		
2:00	—	—	+		
2:20	—	—	+		
2:40	—	—	+		
3:00	—	—	+		
3:20	—	+		2 hrs., 40 min.	0.5
3:40	—	+			0.8
4:00	—	+			0.80
4:20	—	+			0.85
4:40	—	+		4 hrs., 40 min.	1.25
5:00	—	+			1.20
5:20	—	+			1.30
5:40	+				1.30
6:00	+				2.00
6:15	+				2.50

liver is absorbed by the blood capillaries and carried by the blood to the kidneys: "at best the lymphatic system is a secondary factor in the mechanism of jaundice." Bloom in the paper previously mentioned (13) detected bilirubin in blood and lymph by the Van den Bergh technique and observed that a positive test for bilirubin was obtained in the lymph while the blood was still pigment-free. He concluded that "during the first hours after obstruction of the common bile duct, the bile pigments are carried from the liver by the lymph stream and by it are emptied into the general circulation."

Report of Experiments.

The purpose of our second series of experiments was to study the route taken by the direct bilirubin found some hours after obstruction, to reach the blood stream.

The protocol of one experiment is given in detail; the results of the series of experiments have been summarised in Tables III and IV.

TABLE IV.

Dog 11. Experimental Obstructive Jaundice in Nephrectomised Dogs. Van den Bergh Reaction and Bilirubin Estimations in Samples of Blood Serum Withdrawn Every 20 Minutes from the Carotid Artery.

Blood	Van den Bergh reaction			Time after obstruction	Quantitative estimation of bilirubin
Time	Direct	Biphasic	Indirect		
					mg. per liter
12:20	—	—	—		
12:40	Ligation of common and cystic bile ducts				
1:05	—	—	—		
1:20	—	—	—		
1:40	—	—	—		
2:00	—	—	—		
2:20	—	—	—		
2:40	—	—	—		
3:00	—	—	—		
3:20	—	—	+	2 hrs., 40 min.	Traces
3:40	—	—	+		Traces
4:00	—	—	+		0.5
4:20	—	—	+		0.8
4:40	—	+		4 hrs.	1.00
5:00	—	+			1.30
5:20	—	+			1.50
5:40	—	+			1.50
6:00	+			5 hrs., 20 min.	2.00
6:15	+				2.00

Dog 11.—Male, weight 16 kilos. An injection of 3 cg. of morphine was given 39 minutes before ether anesthesia was begun. All the veins entering the left external jugular vein at its junction with the thoracic duct were ligated and a cannula placed in the jugular vein, at 12:15 p.m. The lymph flow was regular at a rate of 20 cc. to 30 cc. per hour. At 12:20 p.m. a cannula was inserted in the right carotid artery and a sample of blood withdrawn. At 12:32 p.m. midline incision and transperitoneal double nephrectomy. At 12:40 p.m. ligation of common and

cystic bile ducts. Samples of blood were withdrawn every 20 minutes, the lymph being examined at the same time. The indirect Van den Bergh reaction appeared first in the lymph 1 hour after the obstruction. 2 hours and 40 minutes after the obstruction the Van den Bergh reaction in the lymph became biphasic, while the indirect reaction appeared in the blood serum. 4 hours and 40 minutes after obstruction the Van den Bergh became direct in the lymph, and 40 minutes later, that is 5 hours and 20 minutes after biliary obstruction, the blood serum showed also the direct Van den Bergh reaction. The dog was killed at 6:20 p.m. with ether. At necropsy the ligatures of bile ducts were well placed. The liver appeared hyperemic. On section the bile ducts were distended with bile. The lymphatics were distended and greenish in colour. Samples of liver were taken for microscopic examinations.

TABLE V.

Experimental Obstructive Jaundice in Dogs Previously Nephrectomised. Ligation of Bile Ducts and Thoracic Duct Fistula.

Dog No.	Sex	Weight kg.	Time of appearance of the Van den Bergh reaction after biliary obstruction in dogs with thoracic duct fistula					
			Lymph			Blood		
			Direct	Indirect	Biphasic	Direct	Indirect	Biphasic
11	M.	16	4 hrs., 40 min.	1 hr.	2 hrs., 40 min.	5 hrs., 20 min.	2 hrs., 40 min.	4 hrs.
14	F.	12.2	4 hrs., 20 min.	50 min.	2 hrs., 25 min.	5 hrs., 15 min.	2 hrs., 30 min.	4 hrs., 15 min.
17	F.	14.4	4 hrs., 50 min.	1 hr.	2 hrs., 50 min.	5 hrs., 45 min.	2 hrs., 55 min.	4 hrs., 30 min.

In one additional experiment the thoracic duct was ligated accidentally. We proceeded as previously, taking samples of blood every 15 minutes. In this dog, the indirect Van den Bergh first appeared in the blood 2 hours and 45 minutes after obstruction. The biphasic reaction appeared 4 hours and 30 minutes and the direct reaction 5 hours and 30 minutes after obstruction. The Van den Bergh reaction in this case behaved as it did in the experiments with thoracic duct fistula.

We summarise in Table V the results of our three experiments, which are in close agreement.

We see from these experiments that after complete biliary obstruc-

tion associated with a thoracic duct fistula, the bile appears first in the thoracic duct before it is detectable in the blood, but that 1 hour later it is present in the blood, not only when the lymph is being drained from the thoracic duct, but even when the duct has been ligated.

Microscopic Examinations of the Liver of These Dogs, 5 to 6 Hours after Total Biliary Obstruction.

It is generally agreed that rupture of bile capillaries appears only in the late stages of biliary obstruction. We made microscopical examinations of the liver of the dogs reported in our experiments. Hematoxylin and eosin sections showed no atrophy or necrosis of the liver cells which appeared quite normal. The bile ducts were not noticeably distended and no bile thrombi were observed. The Kupffer cells in the liver of Dog 8 (whose protocol has been given) contained a moderate amount of brown pigment which could be stained for iron. No such pigment was found in any of the others. Frozen sections stained with Scharlach R revealed only a slight amount of finely divided fat in the liver cells. The cells lining the smaller bile ducts consistently contained fat droplets of various size. Sections were fixed and stained by the method described by Vance (25) for the demonstration of bile canaliculi. The canaliculi were well stained, prominent and slightly distended but the most careful search failed to reveal the rupture of any of them into adjacent lymph spaces.

DISCUSSION.

Taking first in consideration the behaviour of the Van den Bergh reaction in early obstructive jaundice, we see from our experiments that the indirect reaction appears first, then the biphasic and some hours later the direct reaction, which remains during the whole period of obstruction. In spite of the many investigations carried out concerning the nature of the Van den Bergh reaction, it still remains an unsettled problem. Nevertheless from McNee's (26) and Van den Bergh's (27) researches corroborated by Andrews' important work (28), it is clear that the bilirubin normally present in the circulating blood gives only the indirect reaction, and that on passing through the liver cell during excretion, it is changed in some as yet undeter-

mined manner into a form which gives the direct reaction.² What is the significance of the indirect reaction which uniformly appears in the plasma of this animal soon after obstruction, and lasts a short time? It seems to us that a possible explanation for this phenomenon would be that as a consequence of the biliary obstruction a nervous reflex may occur which would lead to sudden paralysis of the function of the liver cells and as a result the circulating bilirubin would remain in the blood stream. This would be analogous to the temporary reflex paralysis of the excretory function of the kidney after ligation of the ureters, which is a well known phenomenon. As the process of bilirubin formation goes on, the bilirubin would soon reach a level sufficient to give a positive test with the diazo reagent. This reflex phenomenon would not last very long. Some hours after obstruction the liver cells would resume their function and the bilirubin giving the indirect reaction would be changed by them into bilirubin giving the direct reaction. We are at present engaged in a study of this possibility.

Another difficult aspect of the problem now presents itself. If in the first hours of obstructive jaundice there is no rupture of bile canaliculi, by what process does the direct reacting bile appear in the blood? In short what is the intimate mechanism of early obstructive jaundice? This is a field in which suggestions only can be made. Must we return to Minkowski's theory as offering the only plausible explanation? The double polarization of the liver cells has been known since Claude Bernard's memorable discovery of the glycogenic function of the liver (29). We know that the epithelial liver cell can function in two different ways: as a gland of internal secretion expelling its products into the blood stream and as a gland of external secretion excreting bile into the bile canaliculi. But it is extremely difficult to comprehend by what mechanism the excretion of bile can shift from an external to an internal secretion.

A close examination of the microscopic sections of the liver in early obstructive jaundice, stained by Vance's technique, shows the bile canaliculi dilated and extending between the liver cells in small distended pouches, the blind end of these lying in contact with the

² In the dog, bilirubin is present in the plasma normally in amounts too small to be detected by the Van den Bergh test.

pericapillary spaces. It would be quite easy to believe that bile might diffuse from these thin walled pouches into the tissue spaces without any actual rupture of the bile canaliculi. This diffusion would be favored by the mounting pressure inside the bile ducts, which rises rapidly according to Bollmann, Sheard and Mann (14).

Regarding the second question, that of the route taken by the bile to reach the blood, since, as we have seen in the thoracic duct fistula experiments, direct reacting bilirubin soon finds its way into the perivascular lymph spaces which surround the hepatic capillaries, bile may diffuse into these capillaries and thus directly enter the blood stream, as we have observed in our experiments.

We think our experiments explain the contradictory results of other investigators concerning the route by which bilirubin reaches the blood in obstructive jaundice. We feel justified in concluding as Wertheimer and Lépage did after their first observations: "*La vérité est que les deux ordres des vaisseaux contribuent à leur resorption et . . . Le rôle des lymphatiques n'en reste pas moins important.*"

CONCLUSIONS.

1. After experimental ligation of the bile ducts in dogs, two distinct processes are clearly manifested: first, the accumulation of the normally circulating bilirubin in the blood with its characteristic indirect Van den Bergh reaction for a period of several hours, and second, the subsequent appearance of the bile bilirubin giving the direct Van den Bergh reaction. It is possible that the first process may be due to a temporary reflex inhibition of the function of the liver cells due to ligation of the duct and comparable to the same phenomenon which usually occurs in the kidney when the ureter is ligated. The second process begins before any rupture of the bile capillaries is visible. Liver sections made 6 to 7 hours after obstruction show these bile capillaries dilated and extending between the liver cells in small distended pouches the blind end of these lying in contact with the pericapillary spaces. It is possible that bile may diffuse from these thin walled pouches into the perivascular lymph spaces, this diffusion being favored by the mounting pressure inside the bile ducts.

2. In early obstructive jaundice bile first appears in the lymph, but exclusion of the thoracic duct from the circulation by drainage causes

a delay of only a few hours in the appearance of bile bilirubin in the blood stream. We must therefore conclude that after biliary obstruction bile enters the circulation both by way of the blood capillaries and the lymphatics, although the latter route is the more important.

We wish to express our thanks to Dr. Arnold R. Rich for his constant assistance and many valuable suggestions.

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PHOTOSENSITIZATION OF ANIMALS AFTER THE INGESTION OF BUCKWHEAT.

By CHARLES SHEARD, Ph.D., HAROLD D. CAYLOR, M.D., AND CARL SCHLOTTHAUER, D.V.M.

(From the Section on Physics and Biophysical Research, the Section on Surgical Pathology, and the Division of Experimental Pathology and Surgery, Mayo Clinic and The Mayo Foundation, Rochester, Minnesota.)

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It has been known for a long time that certain animals, following the ingestion of various substances, are rendered sensitive to certain types of radiant energy. The present study covers biologic and physical observations on the degree of sensitization, the character of the sensitizing light and the nature of the photodynamic substance of buckwheat. White rabbits, mice, rats, goats, swine, dogs and varicolored guinea pigs were studied. Sunlight, carbon arc lamp (Efka or Hoffman) with Conradty-Norris vacuum carbon electrodes and quartz mercury vapor arcs (Victor X-Ray Corporation), both with and without various filters, were employed for irradiation. All of these sources contain infra-red, visible and ultra-violet radiations.

RÉSUMÉ OF LITERATURE.

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Years ago European stockmen found that in certain animals which had ingested buckwheat (plant or seed), erythema, itching, edema and convulsions developed and, in many cases, paralysis and death, on exposure to out-of-door sunshine. However, untoward symptoms did not arise if the animals were kept indoors or in partial darkness and they recovered from the sensitization induced by eating certain plants if they were removed from the light early in the onset of the symptoms. Likewise it was found that the non-pigmented areas of the body were affected readily, whereas the pigmented areas were kept intact. A relatively high degree of intensity of light appears to be a factor in all such diseases as so called buckwheat poisoning (fagopyrismus). Buckwheat causes sensitization to light

which may persist for a few weeks after the last ingestion of this substance provided the animals are kept in a dark place or if cloudy weather intervenes. This is true also after the experimental ingestion of hematoporphyrine.

Finsen observed that such photosensitizations were due to an optically active substance. He studied the influence of daylight on the course of smallpox, in which it would appear that the short or chemical rays must be avoided. He pointed out the similarity between this affection in man and buckwheat poisoning of animals.

At first it was thought that the etiology of fagopyrismus was to be found in secondary organisms, which were believed to live on the plants as parasites, and that these suffered certain changes under the influence of sunlight. Von Tappeiner, however, found in his experiments on photodynamic substances that sickness due to buckwheat could be induced by the removal of fluorescent material from the plant after its exposure to light. Merian arrived at the same conclusions as previous investigators but added the suggestion that the sensitization produced might be due to the longer waves of the visible portion of sunlight. The idea is prevalent in the literature that, since herbivorous animals in their ingestion of food take in considerable quantities of photodynamically active substances, especially chlorophyll, the frequent appearance of diseases due to exogenous sensitization should be expected. Apparently, however, these substances are not taken up by the animal organism in photodynamically active form or they are rendered inactive by some process in the body.

Methods of Experiments.

Feedings.—The goats, rabbits and guinea pigs were fed freshly cut buckwheat and dry seed. The swine received macerated green plant, with milk and bread, in the relative ratio of 2:1. The dogs were fed macerated green buckwheat and chopped meat in equal parts. The rats and mice were fed dry seeds only.

Irradiation.—All animals were kept in cages or stalls away from direct light. During exposure they were placed in a wire mesh cage with an open top. In the investigation of the effects of selective solar irradiation a metallic box was used, over the top of which windows of different absorptive filters were placed as desired. The whole apparatus could be set at angles to bring all animals under the direct rays of the sun.

The Efka carbon arc was operated on 110 volts alternating current and carried 10 amperes. By reason of its construction (with a spherical reflector so angled as to concentrate much of the light and heat from the lamp at a point directly beneath the arc), it was necessary to place the animals in a metallic box built so as to avoid shadows when it was placed about 30 cm. directly in front of the arc proper. Glass filters were fitted in the apertures at the side and top of the box when desired.

An air-cooled quartz mercury vapor lamp was operated at 70 volts and at a distance of 50 cm. from an open topped polished metal box, in which were placed the animals to be irradiated.

The dogs, swine and goats were placed in open areas or fields during irradiation, thus bringing them under a natural environment and permitting slight reactions to be noted.

Observations on Animals after Irradiation.

Sunlight.—After 4 days of the feeding of fresh buckwheat, guinea pigs became photosensitive to direct sunlight. These were the earliest reactions noted. The symptoms, in the order of appearance, were: shaking of the head, excitation, squeaking, scratching of the ears and intense agitation. They attempted to protect themselves by huddling and crawling under each other. The degree of photosensitization increased with continued feeding of buckwheat until, at the end of a week, an exposure of 20 minutes to direct sunlight caused symptoms similar to those in anaphylaxis. The animals were calmed only with complete darkness.

The swine were next in susceptibility. They exhibited marked symptoms of photosensitization on the 10th day of feeding buckwheat. However, the symptoms were not the same as those manifested by the guinea pigs. The reactions noted were: itching, weakness and extensive urticaria followed by sloughing. The first reactions were observed after about 2 hours of direct solar irradiation.

The goats manifested only itching and weakness after 14 days of feeding.

Rabbits, dogs and white rats did not manifest symptoms of photosensitization following direct solar irradiation. The rabbits were exposed to sunlight on every clear day during the feeding of buckwheat for a period of 30 days. No evidences of sensitization were observed even after exposure for 5 hours to direct sunlight. Dogs kept on a diet consisting of 50 per cent buckwheat for 3 weeks were exposed daily to direct sunlight without visible reactions or discomfort, although the periods of exposure were increased to 6 hours. The white rats did not exhibit symptoms of photosensitization.

Quartz Mercury Vapor Lamp.—Irradiation by a quartz mercury lamp did not produce symptoms of sensitization to light in animals fed on buckwheat (plant or seeds). If anything, this type of irradiation appeared to build an additional resistance to photosensitization, probably because an increase in pigmentation was produced.

Carbon Arc.—With the carbon arc, the biologic effects were similar to those produced under direct sunlight but were much less severe.

The Region of Photosensitizing Action.

In order to test whether or not there were any differences in physiologic reactions under various portions of the visible solar spectrum, amber and blue glass filters were used. Light-tight boxes were made and fitted with covers carrying the selected filters. Sensitized animals (guinea pigs) were placed beneath these filters and under direct solar irradiation. Animals under the amber glass (Pittsburgh 48, hammered cathedral) evidenced the same excitability, irritation, itching and scratching as shown by other (or the same) sensitized animals under full sunlight. Animals under blue glass (Pittsburgh 351 or 56, hammered cathedral) remained quiescent and behaved for the most part as did animals having no buckwheat in their diet.

Spectroscopic curves showing the percentages of transmission of light of various wave-lengths by the filters used are shown in Fig. 1. Since the blue filters transmit the region of short wave-lengths (400 to 500 millimicrons) to a maximal amount and since animals fed on buckwheat do not show reaction under such filters, it is apparent that the blue-violet portion of sunlight, together with the near ultra-violet portion also transmitted, is not the photosensitizing region.

Hausmann (1911, 1914) demonstrated that hematoporphyrine has a photodynamic action. It was shown by Freund that eruption in hydroa is produced by wave-lengths from 325 to 396 millimicrons. However, it would appear from the literature that, in experimental sensitization with hematoporphyrine, it is not the invisible but rather the visible portion of the spectrum that is active, namely that in the region of 500 millimicrons.

Certain observers have felt that, by reason of the close parallelism between the chemical and physiologic properties of hemoglobin and chlorophyll and their derivatives (Palladin), such as phylloporphyrine and hematoporphyrine, hematoporphyrine was possibly the photosensitizing agent in the case of buckwheat disease. If the statement can be accepted that the region of 500 millimicrons is the photodynamic portion of the spectrum in the case of hematoporphyrine, then this substance is ruled out of consideration in the present experi-

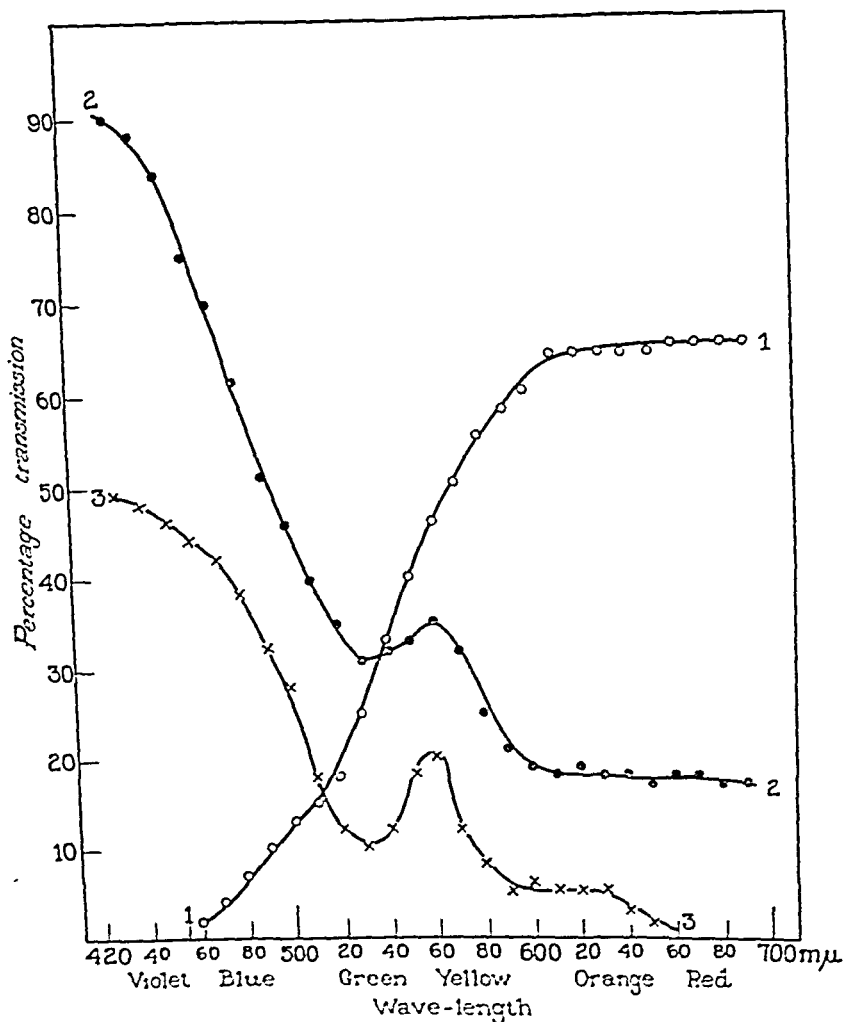


FIG. 1. Spectrophotometric determinations of the percentage transmission of the various filters used. Curve 1, Pittsburgh 48 amber; Curve 2, Pittsburgh 56 blue; Curve 3, Pittsburgh 351 blue glass.

ments for the reason that the energies transmitted by the filters used (Fig. 1) in the region of 500 millimicrons are comparable, yet photodynamic action occurs under the amber filter but not under the blue.

Neither can the difference in physiologic action of the radiant energy transmitted by the different filters be attributed to infra-red radiation. Fig. 2 shows the percentage transmission of the amber and blue filters for wave-lengths of from 750 to 4250 millimicrons. These curves are comparable in all respects; if anything, the blue filter, under which no sensitization occurs, transmits about 10 per cent

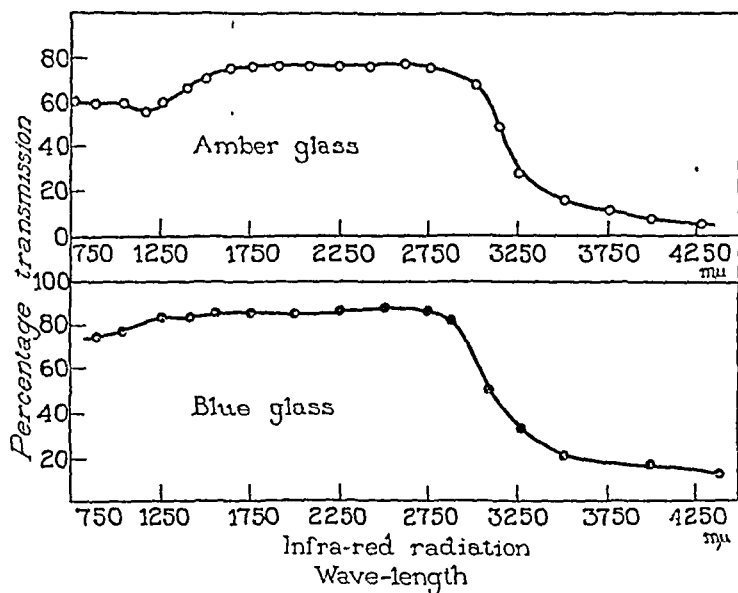


FIG. 2. Curves showing the percentage transmission of amber and blue glass filters for infra-red radiations covering the range of 750 to 4250 millimicrons.

(on the average) more infra-red radiation than does the amber filter (Sheard).

From the character of the physiologic and pathologic reactions produced under various filters and from a consideration of the percentages of transmission of solar energy in the visible spectrum, we conclude that the region of photosensitization lies between 580 millimicrons and the red end of the spectrum.

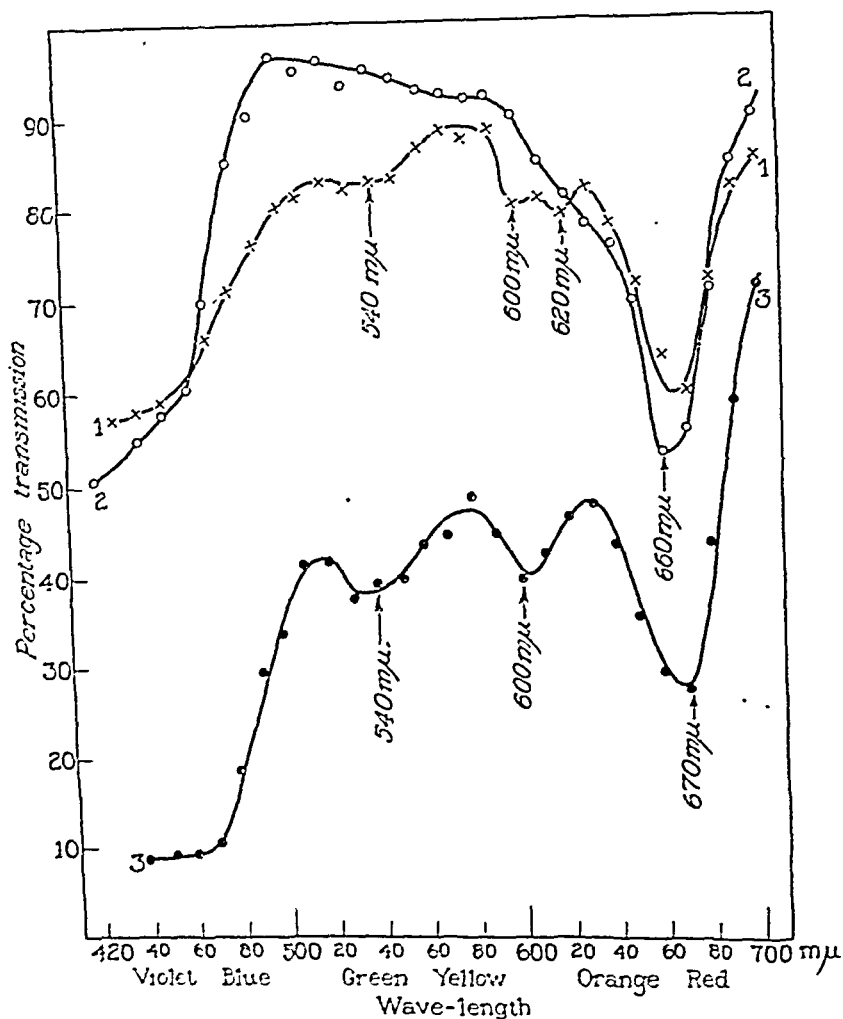


FIG. 3. Spectrophotometric determinations of the percentage transmission of alcoholic extracts of plants. 5 cm. tubes were used in all cases. Curve 1, leaves of buckwheat; Curve 2, grass; Curve 3, stalks of buckwheat.

Spectrophotometric Determinations of Chlorophyll.

From our experience with ethyl alcoholic solutions of chlorophyll (of sufficient dilution) from various sources we believe that such solutions will show two absorption zones, namely, from the limit of the violet end of the visible spectrum to about 500 millimicrons and from 630 millimicrons to approximately 680 millimicrons with a point of maximal absorption at from 660 to 670 millimicrons. That is to say, there is marked absorption in living leaves and in alcoholic solutions of chlorophyll (ethyl chlorophyllide) in the extreme violet and remote red ends of the spectrum. Besides these absorption bands or zones, certain other alcoholic solutions of chlorophyll of equal concentration (as judged by the degree of absorption in the violet and red regions) exhibit additional bands. Curve 2 of Fig. 3 (alcoholic extract from grass) and Curve 1 of the same figure (alcoholic extract of buckwheat leaves) exhibit practical equality of absorption in the regions 430 to 490 millimicrons and 630 to 690 millimicrons. The buckwheat extract, however, exhibits two additional bands with maximal absorption zones at about 540 and 600 millimicrons, as is shown definitely in Curve 3 of Fig. 3 (alcoholic extract of stems of buckwheat). These data may indicate that there are two forms of chlorophyll, such as chlorophyll *a*, and chlorophyll *b*, suggested by Willstätter and Stoll or that there are derivatives of chlorophyll present in certain plants (such as buckwheat) that are not found in other vegetation, such as grass.

The Spectrophotometric Determinations of Blood Serums of Sensitized Animals.

Several attempts were made to obtain satisfactory spectroscopic and spectrophotometric tests on the blood serums of normal and sensitized guinea pigs. Difficulties connected with the prevention of hemolysis of considerable degree, however, did not warrant the drawing of conclusions with respect to the differences in character of the spectrophotometric data, since hemolysis (as evidenced by absorption bands with a maximum at practically 540 and 580 millimicrons) would also cause an increase in spectrophotometric absorption in the blue-violet zone (490 to 430 millimicrons).

The same difficulties likewise existed in general in the serums from swine. In one case, however, serums from a normal and a sensitized pig were obtained relatively free from hemolysis. Curve 1, Fig. 4, shows the spectrophotometric results on the blood serum of a normal pig and Curve 2 contains similar data on the animal photosensitized

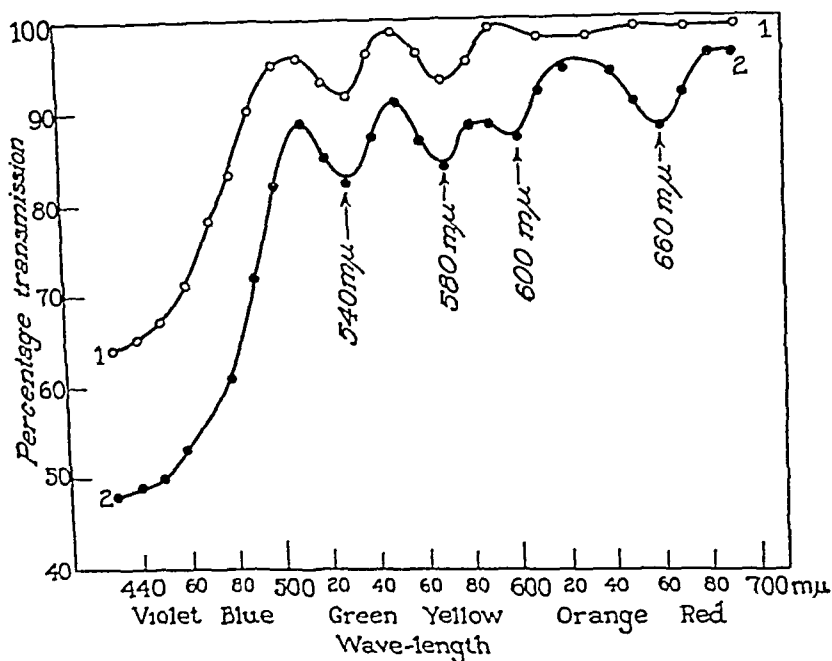


FIG. 4. Spectrophotometric curves showing percentage transmission of light by the blood serum of a normal pig (Curve 1) and by the blood serum of a photosensitized pig (Curve 2).

by buckwheat. Both curves demonstrate the presence of small but practically equal hemolysis, as shown by the absorption bands at 540 and 580 millimicrons. Curve 2, however, shows two additional absorption zones with a maximum at practically 600 and 660 millimicrons, with increased absorption in the blue-violet region (430 to 490 millimicrons).

Pathologic Data.

The anatomic changes noted in cases of buckwheat disease vary according to the length and intensity of the exposure of the sensitized animals. After a short exposure (20 minutes) of a sensitized animal to sunlight, the most noticeable change observed microscopically is hyperemia of the skin, and in most cases accompanying edema. In severe reactions the changes already noted occur and blebs and ulcers may also form. Later, crusts may be formed on the surface. Hemorrhage into the areolar connective tissue may also occur.

Fatal reactions generally demonstrated the anatomic changes mentioned, but there may also be petechial hemorrhage of the lungs, brain, liver, stomach and kidneys. Parenchymatous degeneration is usually found. The most striking feature at the postmortem examination of a photosensitized animal which has died from exposure is the lack of any marked pathologic change. The lesions found suggest the presence of profound toxemia.

In animals sensitized with buckwheat, and exposed to irradiation for a few minutes at a time and over a sufficient number of days, pigmentation involving the basal layer of the skin develops in unpigmented areas. This pigment may be distributed also in the adjacent squamous epithelium and even in the fibrous connective tissue of the deeper layers of the skin. Edema and hyperemia are present also in the skin of those animals having chronic experimental buckwheat disease. The outer layers of the skin are thickened and usually there is free desquamation. Some observers have described hypertrophy of the cartilage of the ears as associated with the hyperkeratosis but it has not been found in our series of animals. In none of the animals observed (not even those exposed to daily periods of irradiation over a period of 6 months) has any lesion developed which we felt suggested a malignant neoplasm.

DISCUSSION.

General.—Urticaria has been noted in domesticated animals pasturing on certain plants. The malady is especially noticeable when white swine pasture on rape and certain clovers. Urticaria on the non-pigmented areas of skin of Holstein and Guernsey cattle is frequently observed. If the animals are kept in darkened stables the

disease soon disappears. Stockmen have attributed the disease to certain insects, dew, nettle rash and plant secretions. Without doubt this affection appears in susceptible animals following the ingestion of certain plants with subsequent exposure to unfiltered sunlight. It may be that many plants are capable of producing this condition and that in ruminating animals the active compound undergoes chemical changes during the long process of fermentation and digestion and is thereby rendered harmless. Therefore, it is possible that photodynamic reactions occur only when large quantities of these plants are ingested and the entire organism becomes saturated.

Pathology.—The anatomic changes noted in sensitized animals that were exposed for brief periods to irradiation are about the same as those found in normal animals after prolonged exposures. Photosensitization therefore accelerates anatomic changes. Following a fatal dosage of light, the anatomic changes (petechial hemorrhages and parenchymatous degeneration) suggest that death is due to profound toxemia which kills the organism before extensive anatomic changes develop. In animals experiencing severe reaction there is, apparently, marked increase in the coagulability of the blood. It is difficult to draw blood from such animals. Postmortem examinations of photosensitized animals, whether killed by the effects of irradiation or by other means, almost always revealed the blood in firm clots in the largest vessels.

The Region of Photosensitizing Action.—Photodynamic action is brought about by the portion of radiant energy which is absorbed by the sensitizing substance. The majority of photodynamic actions which can be attributed to pigment are caused by the visible portions of the spectrum. It follows, therefore, that photodynamic effects are due ordinarily to the longer wave-lengths of visible light. The laws of photochemical reactions apply to photodynamic actions. Temperature has but little effect. The reactions which have been described in this paper were not influenced by extremes of external temperature, for they occurred about as readily on a cool day as on a hot day.

The ultra-violet portion of sunlight, carbon arcs or quartz mercury vapor lamps does not appear to be effective in photosensitization following the ingestion of buckwheat. It is claimed that the presence of hematoporphyrine (which is a derivative of hemoglobin most closely

related chemically to phylloporphyrine, a derivative of chlorophyll) brings about drastic reactions, (under irradiation of wave-lengths from 260 to 400 millimicrons), of the same general character as those produced by buckwheat. Various authorities claim that hydroa æstivale, xeroderma pigmentosum, seaman's skin and smallpox are either caused by or unfavorably influenced by ultra-violet light or the chemically active rays of sunlight (Mayer, pages 167-169). In our experiments, irradiation with ultra-violet light from a quartz mercury vapor lamp did not produce sensitization, but on the contrary built up a resistance to the untoward effects ordinarily produced when buckwheat-sensitized animals were exposed to the longer wave-lengths of sunlight. These observations lead us to believe that (1) exogenous sensitization to light, as induced by buckwheat, is not due to ultra-violet rays, (2) the effect of ultra-violet irradiation in such cases is beneficial, since the development of pigment acts as a protective agent, thereby changing the skin of the animal from a light color to a dark color and (3) hematoporphyrine, in all probability, is not the active substance.

Spectrophotometric Studies of Chlorophyll and of Blood Scrums of Sensitized and Non-Sensitized Animals.—The curves of Fig. 4 we believe demonstrate the presence of chlorophyll or its derivatives in the blood of animals photosensitized by buckwheat. Attempts were made to sensitize dogs to buckwheat but were found to be unsuccessful after a period of daily feeding of buckwheat for over 3 weeks. There is evidence to show that, in all probability, none of the chlorophyll was taken into the blood through absorption by the walls of the digestive tracts of the dogs used in these experiments. Bollman, Sheard and Mann have shown that bilirubin was not formed from chlorophyll under conditions which allowed the formation of bilirubin from hemoglobin. Herbivora excrete chlorophyll in the bile during periods when their rations are rich in chlorophyll. According to Broun, McMaster and Rous, sheep show definitely that chlorophyll may be altered in the body, since a greenish pigment, cholehematin, is produced, when the animal is on green food. This pigment is also excreted in the bile. When cholehematin is administered to dogs it is excreted as such in the bile.

The Nature of the Photodynamic Substance.

According to von Tappeiner, the active principle in photosensitization brought on by buckwheat is an alcohol-soluble fluorescent substance of a nature similar to that of chlorophyllan as described by Hoppe-Seyler. According to Busck, there exists a fluorescent material, fluorophyll, which can be obtained from buckwheat. Alcoholic extracts of buckwheat are said to be inactive in both irradiated and non-irradiated animals. According to Öhmke, such extracts have true fluorescence and the residue obtained from evaporation in a vacuum, when given by mouth to white mice, caused signs of paralysis and even death after the exposure of the animals to light. Von Tappeiner states that the colored material which incites photosensitization is soluble in alcohol.

Fluorescence is a physical property commonly attributed to photodynamic substances or associated with photosensitization. That is, substances producing photodynamic action are able to absorb light and usually fluoresce; the degree of photodynamic action, however, is not proportional to the degree of fluorescence. Only that region of the spectrum in which energy is absorbed by the fluorescent substance (or substances) is effective. It is thought that the presence of a fluorescent or photodynamic substance causes tissues to become susceptible to destruction by the energy contained in wave-lengths of light that would be ineffective otherwise, namely, the longer waves of light, which are able to penetrate deeper into the tissues.

We doubt the necessity of fluorescent action or the need of a fluorescing substance at any point of the photosensitizing process if the reactions produced by the ingestion of buckwheat are to be attributed to the chlorophyll constituent *per se* rather than to some other substance present in buckwheat or to some substance formed in the blood after the ingestion of buckwheat. Our reasons are:

1. Alcoholic and other extracts of grass and buckwheat (presumably largely chlorophyll) give an orange to reddish fluorescence when exposed to irradiation by the violet-blue end of the spectrum. So far as we know, grass is not toxic, whereas buckwheat is.
2. Fluorescence is a phenomenon in which various substances absorb radiation of a certain wave-length (or region) and reemit it as

radiation of a longer wave-length. Fluorescence of chlorophyll in the longer wave-length portion of the spectrum is caused by the absorption of energy below a wave-length of 500 millimicrons. However, exposure of animals after the ingestion of buckwheat to the shorter waves of the visible spectrum does not produce photosensitization. Hence it would appear logical to conclude that the fluorescence of chlorophyll *per se* is not involved in the sensitization brought about by buckwheat.

We venture the suggestion that the photodynamic substance may be phylloporphyrine. The researches of Schunk and Marchlewski have contributed much to an understanding of the chemical character of chlorophyll. The action of hydrochloric acid on a solution of chlorophyll produces, first, chlorophyllan, then phylloxanthin and finally phyllocyanin. Phylloporphyrine is obtained by treating phyllocyanin with strong alkalis. We believe such a substance may possibly be present in the blood of sensitized animals, the hydrochloric acid of the stomach, by its action on chlorophyll, producing chlorophyllan which, on absorption into the blood with an alkalinity represented by pH 7.4, produces phylloporphyrine. Phylloporphyrine is of interest because of its close relationship to hematoporphyrine. The spectrums of the two substances in various solvents are similar. The spectrophotometric curve of the serum of blood from photosensitive animals corresponds in many particulars with that of an alcoholic solution of phylloporphyrine.

It is possible, also, that cholehematin, or its precursor, may be the photodynamic substance. We have a series of experiments under way with reference to this possibility.

SUMMARY.

The chief points presented in this paper are:

1. Following the ingestion of buckwheat (plant or seed) varicolored guinea pigs, white swine and goats exhibited symptoms of photosensitization, the degree of sensitization being in the order given.
2. Rabbits, dogs, white mice and rats did not manifest symptoms of photosensitization.
3. The symptoms and reactions were: agitation, itching, scratching

of the ears, weakness, urticaria with sloughing and symptoms similar to those in anaphylaxis.

4. Microscopic examinations showed the lack of marked pathologic change. The lesions, such as petechial hemorrhage of the lungs, brain, liver, stomach and kidneys, suggest that profound toxemia has been present.

5. Lesions were not found which appeared to be suggestive of malignant neoplasms.

6. Irradiation by a quartz mercury vapor lamp apparently develops a resistance to photosensitization, probably because of increased pigmentation induced by ultra-violet light.

7. From the nature of the physiologic and pathologic reactions produced under various filters and from a consideration of the percentages of transmission of solar energy in the visible spectrum, it would seem that the region of photosensitization lies between 580 millimicrons (yellow) and the red end of the spectrum. This conclusion, moreover, is substantiated by the fact that irradiation by a quartz mercury vapor lamp (which radiates no energy in the visible spectrum at a wave-length greater than 579 millimicrons) produces no symptoms or reactions.

8. Spectrophotometric determinations of alcoholic extracts of grass (non-toxic) and of buckwheat (toxic) show the presence of two additional bands in the absorption spectrum of buckwheat with maxima at about 540 and 600 millimicrons, respectively, together with the common absorption zones at 430 to 490 millimicrons and 630 to 690 millimicrons.

9. Spectrophotometric determinations of blood serums of sensitized animals show, besides the usual absorption bands peculiar to oxyhemoglobin (with maxima at 540 and 580 millimicrons respectively), two zones with maxima at 600 and 660 millimicrons respectively.

10. The fluorescence of chlorophyll *per se*, as suggested by previous investigators, is not, in all probability, the cause of the sensitization induced by buckwheat.

11. Hematoporphyrine is not the photodynamic substance in all probability.

12. Phylloporphyrine may be the photodynamic substance. In this regard, also, the possibility of cholechematin is not to be ruled out.

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